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Roles of Dendritic Cells in Immunity to Toxoplasma gondii

Abstract
Toxoplasma gondii is an intracellular protozoan parasite that actively invades host cells. During toxoplasmosis, dendritic cells (DCs) promote $CD4^+$ and $CD8^+$ T cell responses, which are critical for long-term immunity. Despite this critical role of DCs, questions remain regarding how this population is regulated during infection, and the specific types of interactions (phagocytosis or active invasion) between parasites and DCs that are necessary to induce T cell responses.

Previous studies have observed an infection-induced expansion of DC populations during toxoplasmosis, but the factors that regulate this expansion are currently unknown. Mice deficient in the cytokine Flt3L, which promotes the proliferation and differentiation of DCs and their precursors, were therefore infected with *T. gondii* to determine the role of this cytokine during infection. Flt3L KO mice (which have decreased numbers of DCs) were acutely susceptible to toxoplasmosis, associated with uncontrolled parasite growth, decreased production of the critical cytokines IL-12 and Interferon-gamma, and impaired natural killer cell responses. Infection of these mice induced an expansion of DCs and induced $CD4^+$ and $CD8^+$ T cell proliferation, however these T cells displayed impaired functionality. Administration of exogenous IL-12 prevented acute susceptibility in Flt3L KO mice, thus demonstrating that Flt3L-independent mechanisms contribute to T cell priming and DC expansion during toxoplasmosis, while also highlighting the critical role of DCs as sources of IL-12.

To determine what types of interactions between DCs and parasites lead to T cell responses, a non-replicating strain of *T. gondii* was utilized in combination with a novel approach to track parasite fate in vivo that could distinguish active invasion from phagocytosis, thus enabling the functional and phenotypic properties of infected DCs to be compared with those that had phagocytosed parasites, while controlling for parasite burden. These studies revealed infected DCs to display an activated phenotype unique from DCs that phagocytosed parasites, and to be potent inducers of T cell responses. Furthermore, pharmacological inhibition of invasion abrogated T cell responses, thus demonstrating a critical role for actively infected host cells in inducing adaptive immunity.

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ROLES OF DENDRITIC CELLS IN IMMUNITY TO TOXOPLASMA GONDII

Christopher David Dupont

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ABSTRACT

ROLES OF DENDRITIC CELLS IN IMMUNITY TO TOXOPLASMA GONDII

Christopher D. Dupont

Christopher A. Hunter

Toxoplasma gondii is an intracellular protozoan parasite that actively invades host cells. During toxoplasmosis, dendritic cells (DCs) promote $CD4^+$ and $CD8^+$ T cell responses, which are critical for long-term immunity. Despite this critical role of DCs, questions remain regarding how this population is regulated during infection, and the specific types of interactions (phagocytosis or active invasion) between parasites and DCs that are necessary to induce T cell responses.

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CHAPTER 1: A REVIEW OF RELEVANT LITERATURE

Introduction

Background information on toxoplasmosis and its medical significance

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect any warm-blooded vertebrate, and is a pathogen of medical and veterinary significance (Dubey, 2008). Infection with *T. gondii* can be acquired through congenital infection (Wolf et al., 1939), or through carnivory, if tissue cysts present in the chronically infected host are ingested (Desmonts et al., 1965; Kean et al., 1969). Additionally, it can be acquired through the ingestion of food and water contaminated with parasites in the form of oocysts, which are shed in the feces of infected cats (Frenkel et al., 1970). Following ingestion, the parasite converts to a fast-replicating form known as the tachyzoite, which results in systemic dissemination of the parasite to all tissues. Under normal circumstances this systemic infection is effectively controlled by the host immune response (Johnson, 1992; Weiss and Dubey, 2009). The parasite then converts to a slow replicating form known as the bradyzoite, which persist in tissue cysts in the host neural and muscle tissues for the lifetime of the host (Frenkel, 1973).

The course of infection in humans can range from asymptomatic to severe, depending on the parasite strain and the immune status of the host. The majority of cases of human infection are regarded as asymptomatic and infection rates in some areas are as high as 70% (Pappas et al., 2009). In contrast, congenital infection can result in a number of birth defects, including hydrocephalus, chorioretinitis, intracerebral calcifications, or spontaneous abortion (Havelaar et al., 2007). Toxoplasmosis can also cause severe disease in patients with primary or acquired deficiencies in T cell function, such as those present in patients with AIDS, Hyper IgM Syndrome, those receiving treatment for cancer, and transplant patients being treated with immunosuppressive drugs (Derouin and Pelloux, 2008; Israelski and Remington, 1988, 1993; Leiva et al., 1998; Tsuge et al., 1998; Yong et al., 2008). Although such instances are relatively rare, symptomatic disease in immunocompetent individuals can result from infection with highly virulent strains of *T. gondii* and can cause severe ocular disease or death (Demar et al., 2007; Grigg et al., 2001). In addition to its direct significance to public health, the genetic malleability of

the parasite and its natural ability to infect laboratory animals, have made it an ideal model to study parasite genetics and host-pathogen interactions (Dzierszinski and Hunter, 2008).

Invasion process and intracellular niche

The mechanisms by which *T. gondii* invades host cells and forms an intracellular niche have been extensively reviewed elsewhere (Sibley, 2011), however several aspects of this process are directly relevant to this thesis. *T. gondii* invades host cells through an active process, using it own motor machinery to penetrate the host cell, and phagocytosis of *T. gondii* parasites leads to the killing of the parasite (Sibley, 2011). During invasion, three successive waves of proteins are secreted from parasite organelles, called the micronemes, dense granules, and rhoptries, into the host cell. These proteins can alter host cell function and inhibit the immune response directed towards the parasite (Lim et al., 2012). They also serve to modify the lipid membrane surrounding the parasite, forming a specialized intracellular organelle called the parasitophorous vacuole (PV). The PV allows for the transport of essential nutrients from the host cell to the parasite, while preventing lysosomal fusion, which would lead to the killing of the parasite (Cesbron-Delauw et al., 2008). The sequestered nature of the parasite within the PV raises several fundamental questions regarding the mechanisms by which the parasite interacts with the immune system. For example, can host cells sense the invading parasite, and how would infected cells access parasite antigens for presentation to T cells as is required for the effective control of the parasite.

Parasite virulence

As is the case for many pathogens, the outcome of infection with *T. gondii* is highly dependent on the interplay of host and microbial factors. Genotypic studies have identified three lineages of *T. gondii* into which most strains found in North America and Western Europe can be broadly classified (Howe and Sibley, 1995). In mouse models, parasites belonging to the Type I lineage are highly virulent whereas the Type II and Type III lineages are considered avirulent (Howe and Sibley, 1995; Sibley and Boothroyd, 1992). These differences are also reflected in human

disease, as ocular toxoplasmosis in humans is associated with Type I, but not Type II or Type III strains (Grigg et al., 2001). Given the lethality of Type I strains during murine infection, the vast majority of insights into the mechanisms by which the host immune response controls infection have been gained through studies using avirulent isolates. However, the use of reverse genetics to compare parasite strains that differ in virulence has allowed the identification of secreted *T. gondii* kinases that modify host cell function. Understanding how these parasite enzymes impact host anti-microbial mechanisms provides one approach to define the events that determine the outcome of infection (Weilhammer and Rasley, 2011).

Innate immune responses to *T. gondii*

Following challenge with *T. gondii*, monocytes, neutrophils and dendritic cells (DCs) are recruited to the site of infection, and all of these cell types have been implicated in resistance to this organism (Bliss et al., 2000; Del Rio et al., 2001; Dunay et al., 2008; Dunay et al., 2010; Liu et al., 2006; Mordue and Sibley, 2003; Tait et al., 2010). However, questions remain about their specific roles in controlling infection. One of the most critical functions of the innate immune response to *T. gondii* is the ability to sense the pathogen and produce the cytokine IL-12, which stimulates natural killer (NK) cells and T cells to produce the cytokine Interferon-gamma (IFN-γ) (Gazzinelli et al., 1993b; Gazzinelli et al., 1994; Hunter et al., 1994) (Figure 1.1). IFN-γ is the major mediator of resistance to *T. gondii* and promotes multiple intracellular mechanisms to kill the parasite and inhibit its replication (Table 1.1). This Th1 immune response, defined by the production of IL-12 and IFN-γ, is characteristic of infection with many intracellular pathogens, and as is the case for infection with other intracellular pathogens, mice deficient in either IL-12 or IFN-γ that are infected with *T. gondii* succumb to acute disease and demonstrate an inability to control parasite burden (Gazzinelli et al., 1994; Suzuki et al., 1988).

The innate production of IL-12 during toxoplasmosis requires that the parasite first be sensed by the host. Innate immune receptors called Toll Like Receptors (TLRs) appear to have a role in this process. Thus, mice deficient in the adapter molecule MyD88, which is required for downstream signaling from most TLRs, are acutely susceptible to toxoplasmosis (Scanga et al.,

2002). Specific TLRs implicated in the immune response to *T. gondii* include TLRs 2, 4, 9, 11 and 12. TLRs 11 and 12 respond to a profilin-like molecule conserved among protozoan parasites (Jenkins et al., 2010; Koblansky et al., 2013; Yarovinsky et al., 2005) whereas TLRs 2 and 4 appear to recognize glycosylphosphatidylinositols on the surface of the parasite (Debierre-Grockiego et al., 2007). Additionally, following oral infection with *T. gondii*, bacterial antigens translocate from the gut, and TLRs 2, 4, and 9 respond to these microbial insults, thus contributing to the development of the Th1 immune response (Benson et al., 2009). Although only deficiency in TLR12 results in acute susceptibility to *T. gondii* (Koblansky et al., 2013), the relative contribution of other TLRs is illustrated by the increased cyst burden present in infected mice deficient in one or more of these receptors (Debierre-Grockiego et al., 2007; Yarovinsky et al., 2005). Despite the critical importance of MyD88, other mechanisms of sensing the parasite likely exist, as protective immunity can be induced in MyD88-deficient mice using a vaccine strain of the parasite, and IL-12 responses are not completely abolished in the absence of MyD88 (Scanga et al., 2002; Sukhumavasi et al., 2008).

Numerous studies have aimed to define the primary cell types responsible for production of IL-12 *in vivo* and have identified neutrophils, inflammatory monocytes, macrophages and DCs as relevant sources (Bliss et al., 2000; Bliss et al., 1999a; Gazzinelli et al., 1996a; Mordue and Sibley, 2003; Scanga et al., 2002; Whitmarsh et al., 2011). The relative contribution of DCs to the production of IL-12 during toxoplasmosis has been examined using two mouse models: one in which DCs can be selectively depleted, and another in which DCs specifically lack expression of MyD88 (Hou et al., 2011; Liu et al., 2006). In both cases, altered function or numbers of DCs resulted in lower systemic levels of IL-12 and increased susceptibility to *T. gondii*. In these models, resistance can be restored by treatment with IL-12 or the transfer of wild type DCs, suggesting that DCs are an essential source of IL-12 during toxoplasmosis. Other studies have aimed to define which subsets of DCs are the most relevant sources of IL-12. Following the *in vivo* administration of soluble *T. gondii* antigens, the $CD8\alpha^+$ subset of DCs produces IL-12 (Reis e Sousa et al., 1997). More recently, mice lacking the transcription factor Batf3, which have a deficiency in $CD8\alpha^+$ DCs, have been shown to succumb to *T. gondii* associated with a severe IL-

12 defect, reduced $CD8^+$ T cell responses, and high parasite burdens (Mashayekhi et al., 2011). The finding that exogenous IL-12 restores survival of Batf3 KO mice is consistent with a model in which $CD8\alpha^+$ DCs are an essential source of IL-12. Further evidence for a role for DCs during toxoplasmosis comes from the observations that infection with *T. gondii* induces increased numbers of DCs at the site of infection, in the spleen, and in lymph nodes (Tait et al., 2010) (Mashayekhi et al., 2011). Currently, the mechanisms that regulate this DC expansion are unclear, and they will be explored in Chapter 2 of this thesis.

Neutrophils are another source of IL-12 during toxoplasmosis, as they contain pre-stored IL-12 and can secrete this cytokine *in vitro* and *in vivo* in response to *T. gondii* (Bliss et al., 2000; Bliss et al., 1999a; Bliss et al., 1999b). Additionally, there are reports that neutrophil depletion results in decreased levels of IL-12 and increased parasite replication (Bliss et al., 2001). These findings are complicated by the realization that the strategy used to deplete neutrophils also affects other cell types, including inflammatory monocytes (Dunay et al., 2010). Regardless, mice deficient in the chemokine receptor CXCR2, which is essential for neutrophil recruitment to the site of infection, have higher parasite levels in the central nervous system (CNS), suggesting a role for neutrophils during toxoplasmosis (Del Rio et al., 2001). Neutrophils are also implicated in other effector mechanisms that directly kill parasites, including phagocytosis, the release of reactive chemical species, and the formation of extracellular traps (Abi Abdallah et al., 2012; Chtanova et al., 2008; Konishi and Nakao, 1992; Nakao and Konishi, 1991). While phagocytosis of *T. gondii* by neutrophils has been observed *in vitro* (Konishi and Nakao, 1992; Nakao and Konishi, 1991), several groups have reported that $p47^{phox}$, an enzyme component necessary for the oxidative burst generated by neutrophils following phagocytosis, is unnecessary for resistance to *T. gondii* (Alexander et al., 1997; Egan et al., 2008). Indeed, *in vivo* imaging studies have observed swarms of neutrophils that congregate around infected cells, but the parasites present in the neutrophils appear to be largely intact (Chtanova et al., 2008). However, infection with *T. gondii* does induce increased extracellular DNA at the site of infection, which is dependent upon the presence of neutrophils, and this may be explained by the release of DNA from neutrophils to form extracellular traps (Abi Abdallah et al., 2012). *In vitro* studies suggest that the formation of

these traps results in decreased parasite vitality and may contribute to the control of *T. gondii in vivo*.

Monocytes are also required for resistance during toxoplasmosis, as mice deficient in the chemokine receptor CCR2, which is necessary for monocyte recruitment to the site of infection, exhibit increased susceptibility when challenged (Benevides et al., 2008; Dunay et al., 2010; Robben et al., 2005). Inflammatory monocytes produce IL-12 *in vitro* and *in vivo* when stimulated with *T. gondii*, however it is not clear whether they are a critical source of this cytokine (Aldebert et al., 2007; Benevides et al., 2008; Dunay et al., 2010; Goldszmid et al., 2012; Mordue and Sibley, 2003; Robben et al., 2005; Whitmarsh et al., 2011). It has also been proposed that these populations contribute to the direct control of *T. gondii* through the generation of nitric oxide (NO), which inhibits parasite replication (Dunay and Sibley, 2010). In support of this model, inflammatory monocytes express inducible nitric oxide synthase (iNOS), the enzyme responsible for catalyzing the production of NO, and inflammatory monocytes are able to kill and inhibit the replication of *T. gondii in vitro* (Borges and Johnson, 1975; Mordue and Sibley, 2003; Wilson and Remington, 1979). Additionally, CCR2 KO mice given a low dose oral challenge of *T. gondii* succumb approximately 3-4 weeks after infection, and this is associated with decreased expression of iNOS and increased parasite burdens in the CNS (Benevides et al., 2008). Although monocytes are clearly critical for survival during toxoplasmosis, their role is not limited to production of nitric oxide, as iNOS-deficient mice survive acute challenge, while deficiencies in monocyte recruitment can lead to acute susceptibility (Dunay et al., 2010; Robben et al., 2005; Scharton-Kersten et al., 1997). Monocytes also produce IL-1 in response to soluble toxoplasma antigens (Gazzinelli et al., 1995), and this factor can enhance anti-toxoplasmic effector mechanisms in macrophages and astrocytes *in vitro* (Halonen et al., 1998; Hammouda et al., 1995b). Moreover, IL-1 can synergize with IL-12 to promote production of IFN-γ from innate and adaptive sources (Hunter et al., 1995; Shibuya et al., 1998). It is also possible that monocytes proceed to develop into DCs that are capable of inducing adaptive immune responses (Goldszmid et al., 2012) (Dominguez and Ardavin, 2010), or macrophages that can control

infection through immune GTPase-mediated mechanisms, as will be discussed later in this article.

Natural killer (NK) cells are another innate population involved in immunity to *T. gondii*, and in mice that lack T cells they provide a limited mechanism of resistance through their ability to produce IFN-γ (Denkers et al., 1993; Hunter et al., 1994; Johnson et al., 1993; Sher et al., 1993). NK cell activity peaks early during infection, and although their activity is elevated during chronic toxoplasmosis, they do not appear to be significant contributors to immunity during the chronic stage of infection (Denkers et al., 1993; Hauser et al., 1982; Hunter et al., 1994; Johnson et al., 1993; Kang and Suzuki, 2001; Sher et al., 1993). Consequently, most studies have focused on the early events that control NK cell activity, leading to a model in which IL-12 produced by other innate cells (e.g. neutrophils, monocytes and DCs) promotes NK cell-mediated production of IFN-γ (Gazzinelli et al., 1993b; Hunter et al., 1994). In addition to IFN-γ, NK cells produce the cytokine IL-10, the significance of which will be discussed in a later section of this chapter (Perona-Wright et al., 2009). Human and murine NK cells can also be cytotoxic for cells infected with *T. gondii* (Hauser and Tsai, 1986; Subauste et al., 1992), but it has been proposed that NK cells become infected with the parasite following the lysis of infected cells, which may promote the dissemination of the parasite (Persson et al., 2009).

NK cells can also act to promote adaptive immune responses. Thus, in the absence of $CD4^+$ T cells, they can provide help to the $CD8^+$ T cell response (Combe et al., 2005). One mechanism by which this help is accomplished is by increasing IL-12 production from DCs through interactions dependent on the molecule NKG2D (Guan et al., 2007). Additionally, production of IFN-γ by NK cells has been implicated in the development of optimal $CD4^+$ T cell responses (Goldszmid et al., 2007).

Adaptive Immune Responses to *T. gondii*

The importance of adaptive immune responses for resistance to *T. gondii* during human infection is demonstrated by the increased susceptibility of patients with primary or acquired defects in T cell function, and mice with deficiencies in B cells, $CD4^+$ T cells or $CD8^+$ T cells survive the acute

stage of infection, but ultimately show increased susceptibility to *T. gondii* (Denkers et al., 1997; Johnson and Sayles, 2002; Kang et al., 2000). Understanding how these different cell populations are integrated to provide long-term resistance has been challenging, but several advances in technology have improved our ability to study adaptive immune responses to *T. gondii*. For example, the identification of the molecular epitopes of *T. gondii* recognized by $CD8^+$ T cells has allowed the measurement of antigen-specific $CD8^+$ T cell responses during infection, and provided insight into the mechanisms by which antigen is presented (Blanchard et al., 2008) (Wilson et al., 2010). This has been complemented by the development of parasites that express model antigens such as ovalbumin, β-galactosidase, and EαRFP, as well as the use of two-photon imaging to allow visualization of immune cells following infection (Pepper et al., 2004) (Pepper et al., 2008) (Dzierszinski and Hunter, 2008; John et al., 2010). These advances are currently allowing a higher resolution analysis of the events that mediate the control of *T. gondii*, and enabled many of the experiments performed in this thesis.

$CD4^+$ T cell responses: Initiation and mechanisms of controlling infection

As mentioned earlier, $CD4^+$ T cells are critical for resistance during toxoplasmosis, as the emergence of severe toxoplasmosis is concomitant with the decline in T cell numbers in patients infected with HIV (Israelski and Remington, 1988; Luft et al., 1984). Similarly, in mouse models, the lack of $CD4^+$ T cells is associated with increased susceptibility during the chronic stage of infection (Johnson and Sayles, 2002). $CD4^+$ T cells provide several critical regulatory functions in mediating resistance to toxoplasmosis. During the early stages of infection they contribute to optimal B and $CD8^+$ T cell responses (discussed in subsequent sections of this chapter) (Glatman Zaretsky et al., 2012; Johnson and Sayles, 2002; Lutjen et al., 2006), and the ability of these cells to control chronic infection may be attributed to their production of cytokines such as IFN-γ, or their expression of CD40L (also referred to as CD154), which can activate effector mechanisms in macrophages and other innate cells expressing CD40 on their surface (Andrade et al., 2005; Gazzinelli et al., 1992a; Portillo et al., 2010; Reichmann et al., 2000; Subauste et al., 2007; Subauste and Wessendarp, 2006).

The initiation of T cell responses requires that naïve $CD4^+$ or $CD8^+$ T cells encounter antigen presenting cells bearing their cognate antigen in the context of MHCII or MHCI molecules respectively, in conjunction with co-stimulatory and cytokine signals needed for T cell activation (Curtsinger et al., 2003; Curtsinger et al., 1999; Guermonprez et al., 2002; Harding et al., 1992). During toxoplasmosis, ligation of the molecules CD28 and ICOS, expressed on the surface of T cells, contributes to the co-stimulatory signals, while IL-12 provides the cytokine signal required to promote proliferation and differentiation into populations that produce IFN-γ (Gazzinelli et al., 1994; Villegas et al., 2002; Wilson et al., 2008).

B cells, macrophages, and DCs are all capable of presenting antigen to $CD4^+$ T cells, though DCs are generally considered the most crucial antigen presenting cell population *in vivo* (Jenkins et al., 2001). Following infection with *T. gondii*, multiple populations of DCs undergo expansion and acquire an activated phenotype. Additionally, challenge of mice with parasites engineered to express the model antigen EαRFP revealed that $CD8\alpha^+$ and plasmacytoid DCs (pDCs) express complexes of MHC class II and Eα, a peptide derived from EαRFP, on their surfaces (Pepper et al., 2008). While these studies implicate pDCs and $CD8\alpha^+$ DCs as responsible for presenting antigen to $CD4^+$ T cells during toxoplasmosis, the use of mice with deficiencies in specific DC compartments, as well as mouse models that allow for the selective depletion of DCs or DC subsets, may be useful to further define the roles of these populations in antigen presentation (Jung et al., 2002; Mashayekhi et al., 2011; Takagi et al., 2011).

The mechanisms by which professional antigen presenting cells acquire parasite antigens for presentation in the context of MHCII are unclear, and there are several possible models to explain how this may be accomplished (Figure 1.2). Since there are multiple reports that murine DCs and monocytes infected with *T. gondii* express low levels of MHCII and co-stimulatory molecules, it has been suggested that infected cells would be poor presenters of antigen (Goldszmid and Sher, 2010; Lang et al., 2006; McKee et al., 2004). Thus, antigen acquisition might occur through the phagocytosis of parasites, infected cells, or parasitic debris, or through the endocytosis of antigens secreted by the parasite (Figure 1.2a). *In vitro* studies have demonstrated that murine DCs are able to present antigen derived from live and heat-killed

parasites to $CD4^+$ T cells (Goldszmid et al., 2009). Because heat-killed parasites cannot invade cells, these data are consistent with a model in which antigen is acquired via the phagocytosis of parasites.

Alternatively, antigen may be acquired by antigen presenting cells through active invasion mediated by the parasite, or through abortive invasion events in which antigens are injected into cells that are not subsequently infected (Figure 1.2b) (Koshy et al., 2010). This model is supported by experiments conducted using human monocytes and DCs, in which cells exposed to viable parasites upregulated MHCII and co-stimulatory molecules, whereas cells exposed to heat-killed parasites did not (Subauste et al., 1998; Subauste and Wessendarp, 2000). Additionally, these studies found that exposure to live parasites was necessary for the ability to present antigen. These findings are not entirely consistent with the reports above (Goldszmid et al., 2009; McKee et al., 2004), although this may be attributable to differences between human and murine cells. It is also relevant to note that results obtained from *in vitro* studies may not be representative of what occurs *in vivo*. Currently, *in vivo* studies examining the mechanisms by which antigen is presented to $CD4^+$ T cells during toxoplasmosis are lacking. These issues will be further explored in Chapter 3 of this thesis, in which an in vivo approach is utilized to phenotypically compare the cells that have phagocytosed *T. gondii* to those that are actively invaded. Additionally, pharmacological inhibition of invasion, and the selective transfer of dendritic cells infected with a replication-deficient strain of *T. gondii* will be utilized to determine which of these populations are critical for the induction of adaptive immune responses.

Humoral immunity is essential for resistance to toxoplasmosis

It has long been recognized that infection with *T. gondii* promotes antibody responses, and that these antibodies can kill the parasite (Sabin and Feldman, 1948). Indeed, parasite-specific IgM, IgA, IgE and IgG2 antibodies have been isolated from human patients, and detection of parasite specific antibodies is an effective diagnostic tool to distinguish newly infected individuals from those in the chronic stage of infection (Correa et al., 2007; Remington, 1969; Remington et al., 1968; Remington et al., 2004; Sabin and Feldman, 1948). The critical role of antibody in immunity

to *T. gondii* is demonstrated by the phenotype of μMT mice, which are deficient in B cells. These mice develop apparently normal IFN-γ responses, but succumb to infection within 3-4 weeks following challenge, associated with high parasite burdens in the CNS (Kang et al., 2000). This increase in susceptibility is likely due to a lack of antibodies, as the passive transfer of antibodies confers protection to B cell-deficient mice (Johnson and Sayles, 2002). Antibodies can mediate their protective effects through a variety of mechanisms. *In vitro* studies have found that they can opsonize parasites for phagocytosis, block invasion, and also activate the classical complement pathway (Erbe et al., 1991; Hammouda et al., 1995a; Nakao and Konishi, 1991; Schreiber and Feldman, 1980; Suzuki and Tsunematsu, 1971; Vercammen et al., 1999). The *in vivo* relevance of complement activation is illustrated by studies in which treatment of mice with an antibody that binds to the complement protein C3 results in acute susceptibility to toxoplasmosis (Johnson et al., 1996). Additional studies are required to define the contribution of other antibody-mediated functions.

As mentioned previously, $CD4^+$ T cells are necessary to promote optimal B cell responses and mice deficient in or depleted of $CD4^+$ T cells display lower parasite-specific antibody titers (Glatman Zaretsky et al., 2012; Johnson and Sayles, 2002). Furthermore, the increased susceptibility of $CD4^+$ T cell-deficient mice can be ameliorated by the passive transfer of antibodies, indicating that the defect in antibody responses likely contributes to the failure to control parasite numbers (Johnson and Sayles, 2002). Curiously, infection with *T. gondii* results in severe disruption of splenic architecture and the loss of distinct B cell zones (John et al., 2009) (Glatman Zaretsky et al., 2012). Since B cell zones are considered the main location where $CD4^+$ T cells provide help to B cells (Vinuesa et al., 2005), this raises the question of whether there is a specialized microenvironment where T-B interactions occur when B cell zones are absent. Since disruption of secondary lymphoid structures is characteristic of many infections (Benedict et al., 2006; Cadman et al., 2008; Odermatt et al., 1991; Racine et al., 2010; Scandella et al., 2008; St John and Abraham, 2009), murine models of toxoplasmosis may prove a useful system to interrogate the mechanisms by which $CD4^+$ T cells help B cell responses, and the extent to which splenic architecture contributes to such interactions.

$CD8^+$ T cell response: Initiation and control of parasite burden

Given that *T. gondii* is an intracellular pathogen, it is not surprising that $CD8^+$ T cells, which are specialized to recognize and destroy cells infected with viral, bacterial and parasitic organisms, also have a critical role in mediating resistance to this infection. $CD8^+$ T cells can control infection through the production of inflammatory cytokines such as IFN-γ, through CD40/CD40L interactions, and through the perforin-mediated cytolysis of infected host cells (Denkers et al., 1997; Gazzinelli et al., 1992a; Reichmann et al., 2000). Indeed, mice deficient in $CD8^+$ T cells show increased susceptibility to toxoplasmosis, succumbing approximately 50 days post-infection (Denkers et al., 1997). Furthermore, the adoptive transfer of $CD8^+$ T cells from chronically infected mice, or mice vaccinated with an attenuated strain of *T. gondii*, is sufficient to confer resistance (Gigley et al., 2009; Parker et al., 1991). Additional evidence comes from *in vivo* depletion studies using chronically infected mice, in which depletion of $CD8^+$ T cells and $CD4^+$ T cells results in reactivation of the infection and severe disease, but depletion of $CD4^+$ T cells alone had limited impact (Gazzinelli et al., 1992a).

As previously described, $CD8^+$ T cell responses are initiated when naïve $CD8^+$ T cells encounter their cognate antigen in the context of MHCI on the surface of antigen presenting cells, accompanied by co-stimulatory and cytokine signals. Some of the earliest studies on the $CD8^+$ T cell response identified the Surface Antigen 1 (SAG-1) protein as a target of $CD8^+$ T cells, although the specific peptide sequence of SAG-1 that the $CD8^+$ T cells recognize remains unknown (Khan et al., 1988). More recently, technical advancements have accelerated the discovery of epitopes of *T. gondii* that are recognized by $CD8^+$ T cells. Thus, in 2008, two studies identified peptides derived from *T. gondii* that are presented in the context of the H2-L^d allele of MHCI. These include peptides from the dense granule proteins GRA4 and GRA6, and the rhoptry protein ROP7 (Blanchard et al., 2008; Frickel et al., 2008). Of these, the GRA4 and ROP7 epitopes are conserved across multiple strains of *T. gondii*, whereas expression of the GRA6 epitope is limited to Type II strains. Another epitope, derived from the protein Tgd_057, is presented in the context of the MHCI allele H2-K^b, and is also conserved among multiple genotypic strains (Wilson et al., 2010). The function of Tgd_057 is unclear, but despite the

presence of a secretory signal, it localizes primarily to the cytosol of the parasite. It is of interest that all of these proteins, with the possible exception of Tgd_057, are secreted from the parasite. While this observation likely reflects, in part, the methods used to screen for these epitopes, it is also in agreement with studies in which parasites are engineered to express model antigens, as these studies consistently demonstrate that antigens secreted from *T. gondii* induce more robust T cell responses than antigens expressed in the cytosol (Gregg et al., 2011; Kwok et al., 2003; Pepper et al., 2004). These findings may provide insight into the mechanisms by which antigen is presented to T cells, a theme which will be explored in Chapter 3 of this thesis.

Currently, there are several questions about the identity of the cell populations involved in antigen presentation to $CD8^+$ T cells during toxoplasmosis. DCs are known to be efficient antigen presenting cells, and are crucial for the development of $CD8^+$ T cell responses to *T. gondii*, however there is a lack of studies that clearly distinguish their role as sources of IL-12 from their role as presenters of antigen (Liu et al., 2006; Tait et al., 2010). *In vivo* imaging studies have observed extensive interactions between DCs and antigen-specific $CD8^+$ T cells, suggesting a role for DCs in antigen presentation (Chtanova et al., 2009; John et al., 2009). In contrast, using a bone marrow chimera approach to generate mice in which MHCI expression was limited to non-hematopoietic cells, Dzierszinski *et al*. demonstrated that challenge of these mice with *T. gondii* resulted in an apparently normal $CD8^+$ T cell response (Dzierszinski et al., 2007). One interpretation of these data is that DCs are not necessary for antigen presentation during toxoplasmosis. Further experimentation is therefore necessary to determine which cell type presents antigen to $CD8^+$ T cells.

As is the case for antigen presentation to $CD4^+$ T cells, there are multiple pathways by which parasite antigens may be acquired for presentation to naïve $CD8^+$ T cells (Figure 1.2). *In vitro* and *ex vivo* studies have found that infected DCs are able to present antigen, whereas cells exposed to parasites or parasite antigens that were not infected are unable to do so (Dzierszinski et al., 2007; Goldszmid et al., 2009; Gubbels et al., 2005). In contrast, the DCs observed to interact with $CD8^+$ T cells *in vivo* appear to be largely uninfected (Chtanova et al., 2009; John et al., 2009), suggesting a possible role for uninfected cells in presenting antigen to naïve $CD8^+$ T

cells *in vivo*, which is consistent with numerous reports of cross-presentation in other models of infection (Lin et al., 2008).

While the cellular pathways by which phagocytosed antigens are processed and eventually presented in the context of MHCI have been widely studied in a variety of systems (Lin et al., 2008), it is less clear how a cell infected with *T. gondii* can present antigen, given that the parasite resides in a specialized non-fusogenic vacuole. Several studies using reporter systems in which host cells respond to antigens derived from *T. gondii* have demonstrated that secreted antigens can enter the cytoplasm of infected cells (Gubbels et al., 2005; Koshy et al., 2010). These antigens would then be transported from the cytosol into the endoplasmic reticulum by the Transporter Associated with Antigen Processing (TAP) (Gubbels et al., 2005). This model is consistent with studies demonstrating that secreted antigens from *T. gondii* are preferentially presented to T cells (Blanchard et al., 2008; Frickel et al., 2008; Gregg et al., 2011; Kwok et al., 2003; Pepper et al., 2004). Alternatively, the PV can fuse with the endoplasmic reticulum, providing another mechanism by which antigens may escape sequestration and enter the protein transport pathway (Goldszmid et al., 2009). The role of infected cells in initiating $CD8^+$ T cell responses during toxoplasmosis will be further explored using an in vivo model in Chapter 3 of this thesis.

$CD8^+$ T cell responses to *T. gondii* are influenced by help provided by $CD4^+$ T cells (Combe et al., 2005; Lutjen et al., 2006). Although depletion of $CD4^+$ T cells does not affect the magnitude of the $CD8^+$ T cell response during the early stage of $CD8^+$ T cell expansion and activation, $CD4^+$ T cells are necessary for the maintenance of $CD8^+$ T cell effector functions during the chronic stage of infection, and this help must be provided during the acute stage of infection (Lutjen et al., 2006). Further insights regarding the nature of $CD4^+$ T cell help have been gained from studies using the attenuated vaccine strains of *T. gondii* ts-4 and *cpsII*, both of which require $CD4^+$ T cell help for optimal protective $CD8^+$ T cell responses (Denkers et al., 1996; Jordan et al., 2009). In current models, ts-4 vaccination stimulates $CD4^+$ T cells to produce the growth factor IL-2, which provides an essential signal for $CD8^+$ T cells. Indeed, neutralization of IL-2 results in diminished $CD8^+$ T cell responses and decreased protection (Denkers et al., 1996).

Other potential mechanisms by which $CD4^+$ T cells may provide help include the licensing of DCs, or direct interactions with $CD8^+$ T cells through CD40/CD40L interactions (Bevan, 2004).

The vast majority of studies examining the $CD8^+$ T cell response have used avirulent Type II strains of *T. gondii*. Recently, it has become apparent that $CD8^+$ T cell responses are dramatically decreased following infection with the highly virulent RH strain of the parasite and there are several possible explanations for this phenotype (Tait et al., 2010). The defective $CD8^+$ T cell response may be influenced by the activities of the parasite virulence factor ROP18 which (in addition to other functions) binds to the host protein Activating Transcription Factor 6 β (ATF6β), leading to its degradation (Yamamoto and Takeda, 2012). In support of this model, ATF6β-deficient mice have a defective $CD8^+$ T cell response when infected with *T. gondii*, and ROP18-deficient parasites from an RH background induce greater production of IFN-γ from $CD8^+$ T cells, relative to wild type RH parasites. Decreased $CD8^+$ T cell responses may also result from an abbreviated DC response during RH infection, relative to infection with an avirulent Type II strain (Jordan et al., 2010). As the adoptive transfer of large numbers of antigen-specific $CD8^+$ T cells is able to transiently reduce parasite burden during RH infection, it seems likely that the decreased $CD8^+$ T cell response is a contributing factor to the virulence of the RH strain (Tait et al., 2010).

More subtle changes in $CD8^+$ T cell responses may also help to explain differences in susceptibility among mouse strains. Whereas the C57B/6 inbred mouse strain succumbs to *T. gondii* during the chronic stage of infection, BALB/c mice are relatively resistant to toxoplasmic encephalitis. This difference in susceptibility has been genetically mapped to the MHC Class I H2-L^d allele, implicating $CD8^+$ T cells as being responsible for this difference in susceptibility (Brown et al., 1995; Suzuki et al., 1994). The recent identification of an immunodominant epitope from the protein GRA6, recognized by $CD8^+$ cells, that binds to the H2-L^d Allele has led to the hypothesis that recognition of this peptide is crucial for controlling *T. gondii* infection in BALB/c mice, and may account for the differences in virulence among mouse strains (Blanchard et al., 2008). Because expression of this epitope is restricted to Type II strains of *T. gondii*, its relative significance could be tested by replacing the peptide with the sequence present in Type I or Type

III strains. Alternatively, it may be possible to tolerize mice to this epitope through vaccination, as has been reported in other systems (Rosenberg et al., 2010). Regardless, these studies highlight the importance of GRA6 as a target for protective CD8+ T cells.

Effector mechanisms controlling *T. gondii* infection

As discussed in the previous section, cellular immunity mediates protection through the production of inflammatory cytokines such as IFN-γ. Other molecular signals, such as the cytokine tumor necrosis factor alpha (TNF-α) and CD40 ligation are also required for resistance during chronic toxoplasmosis (Deckert-Schluter et al., 1998; Reichmann et al., 2000; Schluter et al., 2003; Yap et al., 1998). This section describes how these distinct pathways are integrated to engage specific effector mechanisms required to directly control infection with *T. gondii*.

Nitric oxide inhibits replication of *T. gondii*

Since the early 1980's, it was recognized that IFN-γ can activate macrophages to kill a variety of intracellular organisms, including *T. gondii* (Nathan et al., 1983), and during the late 1980's it was reported that IFN-γ is also essential *in vivo* for resistance to *T. gondii* (Suzuki et al., 1988). These findings raised the fundamental question of how this cytokine promotes control of *T. gondii* and other pathogens. It was proposed that the protective effects of IFN-γ may be mediated by inducing increased synthesis of Nitric Oxide (NO) (Adams et al., 1990). Consistent with this hypothesis, expression of inducible nitric oxide synthase (iNOS), the enzyme responsible for catalyzing the reaction that results in production of NO, is increased in macrophages by stimulation with IFN-γ, and NO inhibits replication of *T. gondii* in macrophages and other cell types (Chao et al., 1993; Jun et al., 1993; Koide et al., 1993; Langermans et al., 1992; Zhao et al., 2009a). Importantly, IFN-γ alone is not typically sufficient to activate macrophages to kill *T. gondii*, and additional signals provided by factors such as TNF-α or CD40L are required for optimal iNOS expression (Jana et al., 2001; Langermans et al., 1992). *In vivo* evidence for a role of NO in controlling toxoplasmosis came from a study in which administration of the iNOS inhibitor aminoguanidine to infected mice resulted in increased parasite burdens (Hayashi et al.,

1996). Subsequently, iNOS-deficient mice were developed and found to display increased susceptibility to toxoplasmosis, succumbing to disease in the chronic stage of infection (Scharton-Kersten et al., 1997). Although the specific mechanism by which NO inhibits replication of *T. gondii* remains to be determined, studies using intracellular bacterial pathogens have shown that NO can inhibit bacterial enzymatic activity and directly damage DNA (Chakravortty and Hensel, 2003), which would preferentially affect pathogen replication and account for the static effects of NO.

IFN-γ mediates protection through the p47 GTPases

The increased susceptibility of iNOS-deficient mice to toxoplasmic encephalitis clearly implicated iNOS in immunity to *T. gondii*, but also pointed toward iNOS-independent mechanisms by which IFN-γ mediates protection during the acute phase of infection. Like iNOS, members of the p47 GTPase family (also referred to as the Immune Related GTPase family (IRGs)) are also upregulated in response to IFN-γ (Howard et al., 2011; Taylor et al., 1996), but the importance of this family was first apparent when mice that lack the p47 GTPase IGTP (Irgm3) were infected with *T. gondii*. These mice have normal IFN-γ responses, but succumb to acute toxoplasmosis due to high parasite burdens (Taylor et al., 2000). Subsequent studies revealed other members of this family, including LRG-47 (Irgm1), IRG-47 (Irgd), IIGP1 (Irga6), and TGTP (Irgb6) to be involved in immunity to *T. gondii* as well (Collazo et al., 2001; Fentress et al., 2010; Pawlowski et al., 2011; Zhao et al., 2009a). The specific mechanisms by which individual members of the p47 GTPase family promote the clearance of *T. gondii* are the subject of ongoing studies in many laboratories (Howard et al., 2011). There are reports that in IFN-γ activated cells p47 GTPases colocalize to the PV, which then develops a tight fitting morphology followed by a rough and disrupted appearance before being stripped away (Fentress et al., 2010; Hunn et al., 2008; Khaminets et al., 2010; Ling et al., 2006; Martens et al., 2005; Steinfeldt et al., 2010; Zhao et al., 2009a; Zhao et al., 2009b; Zhao et al., 2008). Once free in the cytosol, the parasite egresses the infected cell or becomes permeabilized and killed (Melzer et al., 2008; Zhao et al., 2009a). In the latter studies, the host cell was observed to undergo necrosis after killing the parasite.

Additionally, other studies have observed the exposed cytosolic parasite to be disposed of by xenophagy, the process by which foreign bodies within a cell are eliminated using the same cellular machinery involved in autophagy (Ling et al., 2006). In further support of a role for autophagic machinery in immunity to *T. gondii*, the autophagy protein Atg5 has been found to be necessary for the disruption of the PV and resistance to this infection *in vivo* (Zhao et al., 2008). Additionally, CD40 ligation has been observed to induce xenophagic elimination of parasites independently of p47 GTPases, as will be discussed a later section of this chapter (Subauste and Wessendarp, 2006).

Given the important role of the p47 GTPases in immunity to *T. gondii*, it is not surprising that the parasite has evolved strategies to interfere with their function. At least three members of the p47 GTPase family, Irga6, Irgb6 and Irgb10, are phosphorylated by ROP18, resulting in changes in their functionality or cellular localization associated with increased virulence (Fentress et al., 2010; Steinfeldt et al., 2010). Additionally, the recruitment of GBP1, a member of the guanylate-binding protein family (GBPs), to the PV is also inhibited by the parasite-derived virulence factors GRA15, ROP16 and ROP18 (Virreira Winter et al., 2011), and recent reports have demonstrated that this family of enzymes participates in p47 GTPase-mediated killing (Selleck et al., 2013; Yamamoto et al., 2012).

The role of tryptophan degradation as a defense mechanism

IFN-γ can also mediate protective effects against *T. gondii* by promoting tryptophan degradation in a variety of infected cell types, including fibroblasts, macrophages, and brain cells (Daubener et al., 1996; Daubener et al., 2001; Murray et al., 1989; Pfefferkorn, 1984). Treatment of cells with IFN-γ results in the upregulation of the genes indolamine 2,3-dioxygenase 1 and 2 (IDO-1 and IDO-2), which catalyze the degradation of tryptophan (Metz et al., 2007; Murray et al., 1989). Because *T. gondii* is a natural tryptophan auxotroph, the increased degradation of tryptophan by host cells inhibits parasite growth (Sibley et al., 1994). The *in vivo* relevance of this pathway is illustrated by the finding that long-term treatment of infected mice with inhibitors of IDO-1 and 2 results in increased susceptibility and increased parasite burdens during chronic infection

(Divanovic et al., 2012). Interpretation of this finding is complicated by the fact that IDO has other known immune functions such as suppression of DC and effector T cell functions, as well as promotion of regulatory T cell responses (Soliman et al., 2010).

Members of the TNF family are necessary for immunity to *T. gondii*

In addition to IFN-γ, members of the TNF family such as CD40L, TNF-α and LT-α, are also required for protection during the chronic stage of infection (Reichmann et al., 2000; Schluter et al., 2003; Yap et al., 1998). The critical role of TNF-α is demonstrated by studies in which neutralization of this cytokine results in increased susceptibility and higher parasite burdens (Gazzinelli et al., 1993a). Additionally, mice deficient in TNF-α (TNF-α KO) or the components of its receptor (TNFR KO) succumb to infection approximately 3-4 weeks post-challenge despite having functional IFN-γ responses (Deckert-Schluter et al., 1998; Schluter et al., 2003; Yap et al., 1998). TNF-α is produced by a number of cell populations in response to *T. gondii* or *T. gondii* antigens, including neutrophils (Bennouna et al., 2003; Bliss et al., 1999a), DCs (Bennouna et al., 2003), macrophages (Li et al., 1994), microglia (Schluter et al., 2001), and T cells (Schluter et al., 1997). TNF-α synergizes with IFN-γ to promote anti-parasitic mechanisms in macrophages, as well as non-hematopoietic cells (Chang et al., 1990; Yap and Sher, 1999). *In vitro* studies have demonstrated that this can be mediated through the production of nitric oxide (Koide et al., 1993; Langermans et al., 1992). Additionally, TNF-α KO mice, TNFR KO mice, and mice treated with a neutralizing antibody for TNF-α display decreased iNOS expression (Deckert-Schluter et al., 1998; Gazzinelli et al., 1993a; Schluter et al., 2003). Collectively, these data support a model in which TNF-α mediates its protection by inducing expression of iNOS. However, there are also data that suggest that susceptible TNFR KO mice infected with *T. gondii* can have appropriate levels of iNOS, indicating that TNF-α can mediate protection through iNOS-independent mechanisms (Yap et al., 1998). Because TNF-α KO and TNFR KO mice are capable of surviving the acute stage of infection, it is clear that TNF-α is not required for the IGTP-mediated elimination of the parasite (Deckert-Schluter et al., 1998; Schluter et al., 2003; Yap et al., 1998). This notion is also supported by *in vitro* studies, in which macrophages show no defect in their

ability to kill parasites in the absence of TNF-α signaling (Zhao et al., 2007). However, interpretation of these results is complicated by the finding that TNF-α plays a more prominent role in activating macrophages when concentrations of IFN-γ are limiting (Yap et al., 1998). Thus, the chronic susceptibility of mice deficient in TNF-α signaling may result from changes in the expression of IFN-γ during the course of infection rather than a deficiency in any one specific effector mechanism that is absolutely dependent upon TNF-α.

Another component of the TNF family involved in immunity to *T. gondii* is CD40L, which is expressed on T cells and binds to CD40 expressed on macrophages and other cell populations (Subauste, 2009). The importance of CD40/CD40L interactions to promote immunity to *T. gondii* is evidenced by the increased susceptibility of patients with Hyper-IgM syndrome, a disease characterized by defective CD40L expression (Leiva et al., 1998; Tsuge et al., 1998; Yong et al., 2008). During human toxoplasmosis, CD40/CD40L interactions are necessary to promote optimal production of IFN-γ and class switched antibody (Leiva et al., 1998; Subauste et al., 1999). In contrast, these interactions are not critical for production of IFN-γ in the murine model, yet mice deficient in CD40L display increased susceptibility during chronic infection (Reichmann et al., 2000). While CD40L can act synergistically with IFN-γ to inhibit parasite replication, there is also evidence that CD40L can act independently of IFN-γ (Andrade et al., 2005; Portillo et al., 2010; Reichmann et al., 2000; Subauste and Wessendarp, 2006). One IFN-γ independent mechanism by which CD40L controls infection is through the induction of xenophagic killing of the parasite, which has been shown to be independent of the p47 GTPase family, but dependent upon the autophagic molecule Beclin-1 (Portillo et al., 2010; Subauste et al., 2007; Subauste and Wessendarp, 2006). Beclin-1-heterozygous mice also demonstrate increased susceptibility to *T. gondii* infection, indicating that CD40-mediated xenophagy may be a unique and critical mechanism for controlling chronic toxoplasmosis.

Lymphotoxin alpha (LT-α) is another member of the TNF family essential for immunity to *T. gondii*. Like TNF and TNFR KO mice, LT-α KO mice succumb to this infection within the first 4 weeks, associated with a high parasite burden (Schluter et al., 2003). These mice display functional but delayed IFN-γ responses and antibody titers, and decreased expression of iNOS.

These defects may conceivably result from a critical role for LT-α in signaling to directly promote effector functions, or they may be a secondary consequence of the defective splenic architecture observed in LT-α KO mice (De Togni et al., 1994).

Thus, cytokines and the effector mechanisms they induce are able to control toxoplasmosis, allowing the parasite and the host to co-exist. Parasite virulence factors or immunodeficiency can disrupt this equilibrium, leading to severe disease or the death of the host. However, the immune effector mechanisms that control parasite burden can also bear a fitness cost upon the host, as will de described in the following section.

Severe immunopathology and the mechanisms that prevent it

As is true for many infections, maintaining immune homeostasis during toxoplasmosis requires not only the ability to limit the replication of the pathogen, but also the ability to control the host immune response. In wild type mice this is illustrated by severe infection-induced inflammation in the gut and central nervous system that is mediated by $CD4^+$ T cells. This section will review the factors that contribute to these pathologic events and the mechanisms by which they are controlled.

Intestinal Ileitis following infection with *T. gondii*

Immunopathology can occur in the ileum following oral infection with *T. gondii* in mice and other species, and this ileitis has been proposed as a model to understand the basis for immune-mediated gastrointestinal disease in humans (Egan et al., 2011a; Liesenfeld et al., 1996). The infection-induced ileitis is characterized by the development of severe necrosis and inflammatory foci, and is dependent upon the host's sex and genetic background (Liesenfeld et al., 1996; Liesenfeld et al., 2001). That this process is immune mediated is demonstrated by studies in which C57B/6 mice lacking $CD4^+$ T cells or mice depleted of $CD4^+$ T cells fail to develop this phenotype (Liesenfeld et al., 1996). Development of the ileitis is a complex process involving numerous cell types, including intraepithelial lymphocytes, natural killer T cells, and NK cells (Egan et al., 2009a; Egan et al., 2011b; Khan et al., 2006; Ronet et al., 2005). Factors that

promote T cell responses, such as CD40/CD40L interactions and the cytokines IL-12 and IL-23 also contribute to ileitis development (Li et al., 2002; Munoz et al., 2009; Vossenkamper et al., 2004). Other cytokines, such as IFN-γ, TNF-α, IL-18, IL-22, and the macrophage migration inhibitory factor (MIF) have also been implicated in mediating pathology (Cavalcanti et al., 2011; Liesenfeld et al., 1999; Munoz et al., 2009; Vossenkamper et al., 2004). The Th2 cytokines IL-4 and IL-5 have also been implicated in ileitis development, although another report found IL-4-deficient mice to be more susceptible to oral challenge (Nickdel et al., 2004; Nickdel et al., 2001; Roberts et al., 1996).

As work on this model has progressed, it has become clear that the commensal bacteria present in the gut contribute to the development of this infection-induced ileitis (Heimesaat et al., 2006). Recent findings have led to a model in which oral challenge with *T. gondii* results in a dramatic increase in the quantity of Gram-negative bacteria in the gut flora and bacterial translocation to subepithelial tissues (Heimesaat et al., 2006) where TLR4 senses these bacteria and amplifies the local inflammation (Heimesaat et al., 2007). Additionally, mice deficient in TLR11, which binds to profilin expressed by the parasite, do not develop ileitis, suggesting that the innate response to *T. gondii* also contributes to this process (Benson et al., 2009). While these findings implicate both parasitic and bacterial antigens in stimulating the pathologic immune response, the antigen specificity of the $CD4^+$ T cells that mediate ileitis remains to be determined.

$CD4^+$ T cell mediated immunopathology during Toxoplasmic encephalitis

Another example of severe pathology occurs in mouse models of chronic toxoplasmic encephalitis. Although $CD4^+$ T cells are essential for long-term resistance to *T. gondii*, they also can induce severe pathology in the central nervous system. Thus, in susceptible mice large numbers of $CD4^+$ T cells are present in the brain during the chronic encephalitis, and depletion of $CD4^+$ T cells can ameliorate pathology without affecting parasite burden (Israelski et al., 1989). Similarly, CD28 KO mice infected with *T. gondii* have normal parasite burdens but exhibit enhanced resistance to toxoplasmic encephalitis that correlates with decreased numbers of $CD4^+$ T cells in the brain (Reichmann et al., 1999). This work contrasts with studies in which depletion

of $CD4^+$ T cells was sufficient to reactivate disease (Vollmer et al., 1987). These seemingly conflicting results may be partially explained by differences in depletion efficiency, as complete depletion of $CD4^+$ T cells in the central nervous system can be difficult to obtain, and is not necessarily reflected by the depletion efficiency in other tissue sites (Wilson et al., 2005). Thus, while $CD4^+$ T cells are required for control of infection, partial inhibition of $CD4^+$ T cell responses may be beneficial to the host in the context of chronic toxoplasmic encephalitis.

IL-10 inhibits $CD4^+$ T cell mediated immunopathology

Since 1996, the use of various knockout mice has led to the identification of factors critical for limiting the development of immune pathology during toxoplasmosis. These studies have provided a novel insight into the nature of host-pathogen interactions, which is perhaps best illustrated by studies in which mice deficient in the cytokine IL-10 (IL-10 KO) were challenged with *T. gondii* (Gazzinelli et al., 1996b). IL-10 is produced by a number of cell types, including macrophages, NK cells, T cells and B cells, and functions by inhibiting the activation of accessory cells and adaptive immune responses (Moore et al., 2001). The central role for IL-10 in limiting inflammation was confirmed by the finding that IL-10 KO mice develop spontaneous colitis (Kuhn et al., 1993). However, IL-10 is also a potent antagonist of the ability of macrophages to kill intracellular bacteria and parasites, such as *T. gondii*, and infection with a number of pathogens, including *T. gondii*, increases expression of IL-10 (Gazzinelli et al., 1993a; Gazzinelli et al., 1992b; Hunter et al., 1993; Khan et al., 1995; Sher et al., 1992). These findings led to the idea that pathogens induce IL-10 production as a means to evade the immune response (Sher et al., 1992). However, challenge of IL-10 KO mice with *T. gondii* revealed that these mice display normal parasite burdens, but develop severe liver damage, increased production of pro-inflammatory cytokines, and succumb to a $CD4^+$ T cell mediated hyper-inflammatory response (Gazzinelli et al., 1996b). These results provide one of the first examples of an infection in which the host must control its own immune response and tolerate pathogen persistence in order to survive.

While IL-10 is clearly critical for survival during the acute stage of infection, studies analyzing the role of IL-10 during toxoplasmic encephalitis have yielded more ambiguous results. There is general agreement that IL-10 expression is upregulated in the brain during chronic toxoplasmosis, and macrophages and $CD4^+$ T cells represent a local source of IL-10 in the central nervous system (Deckert-Schluter et al., 1997; Wilson et al., 2005). One study observed that neutralization of IL-10 during chronic toxoplasmosis was not lethal to infected mice, and resulted in decreased parasite burden (Deckert-Schluter et al., 1997). In contrast, another study reported that blocking of the IL-10R resulted in decreased survival of chronically infected mice (Jankovic et al., 2007). Similarly, in a system that allowed IL-10 KO mice to survive acute infection, the chronically infected IL-10 KO mice displayed normal parasite burden but also developed severe immunopathology mediated by $CD4^+$ T cells associated with increased production of pro-inflammatory cytokines (Wilson et al., 2005). Together, these results suggest that while IL-10 may partially inhibit effector mechanisms that could otherwise reduce parasite burden, it is crucial in the acute and chronic stage of infection to prevent severe immunopathology.

Attempts to identify the cellular sources of IL-10 during toxoplasmosis have revealed that there are multiple contributors, including macrophages (Deckert-Schluter et al., 1997), NK cells (Perona-Wright et al., 2009), $CD4^+$ T cells (Deckert-Schluter et al., 1997; Jankovic et al., 2007) and $CD8^+$ T cells (Deckert-Schluter et al., 1997). Innate sources of IL-10 are significant during toxoplasmosis, as the loss of IL-10 expression from SCID mice can dramatically extend their survival (Neyer et al., 1997), and NK cells are regarded as a major innate source of IL-10 during the acute stage of infection (Perona-Wright et al., 2009). Nevertheless, the use of a cre-lox system to selectively eliminate IL-10 production from $CD4^+$ and $CD8^+$ T cells revealed that these mice still develop severe immunopathology upon infection (Roers et al., 2004), indicating that non-T cell sources of IL-10 were not sufficient to limit pathology. Subsequent work has demonstrated that depletion of $CD4^+$ T cells dramatically decreases expression of IL-10, and that the $CD4^+$ T cells that produce IL-10 express the transcription factor T-bet and low levels of CD25, which was interpreted to suggest that they were effector T cells as opposed to regulatory T cells

(Jankovic et al., 2007). However, the recent identification of a population of T-bet$^+$ Foxp3$^+$ population of regulatory T cells that produce IL-10 during toxoplasmosis calls this interpretation into question (Hall et al., 2012).

IL-27 inhibits CD4$^+$ T cell mediated immunopathology

IL-27 is another cytokine critical for regulating CD4$^+$ T cell responses during toxoplasmosis. Initial reports concluded that the IL-27 receptor was important in promoting Th1 immune responses (Watford et al., 2003), however when mice deficient in WSX1, a component of the IL-27 receptor, were infected with *T. gondii*, these mice were found to exhibit enhanced Th1 responses and dramatically increased susceptibility to this challenge (Villarino et al., 2003). Similar to the IL-10 KO mice, infected WSX1 KO mice have normal parasite burdens but develop severe liver and lung pathology, and increased numbers of activated CD4$^+$ and CD8$^+$ T cells. Furthermore, depletion of CD4$^+$ T cells prevents the infection-induced pathology. Although initial studies indicated that the suppressive effects of IL-27 were independent of IL-10 (Villarino et al., 2003), subsequent work has demonstrated that IL-27 promotes IL-10 expression (Stumhofer et al., 2007). However, this property of IL-27 does not fully explain its suppressive activities. IL-27 can also act directly on CD4$^+$ T cells to inhibit production of IL-2, a growth and survival factor for T cells, and neutralization of IL-2 results in enhanced survival of WSX1-KO mice (Villarino et al., 2003). Another recent study found that deficiency in EBI3, a component of IL-27, correlated with decreased expression of the inhibitory molecule PD-L1 on CD4$^+$ T cells during toxoplasmosis, providing another potential mechanism by which IL-27 may mediate its protective effects (Hirahara et al., 2012).

Although WSX1 KO mice normally do not survive to chronic infection, treatment with anti-toxoplasma drugs or immune blockade can prevent acute susceptibility, allowing the role of IL-27 during chronic infection to be examined (Stumhofer et al., 2006). Under these circumstances, WSX1 KO mice show no defect in their ability to control parasite burden but do develop severe immunopathology in the central nervous system, which correlated with decreased production of IL-10 and increased production of IL-17 (Stumhofer et al., 2006; Stumhofer et al., 2007). These

findings led to the recognition that IL-27 can directly inhibit production of IL-17 from Th17 cells (Stumhofer et al., 2006). These results collectively support a model in which IL-27 controls immunopathology during toxoplasmosis by inhibiting multiple facets of T cell activation, and the principles established using *T. gondii* have been shown to be relevant to the immunosuppressive effects of IL-27 in a variety of systems, including infections with other intracellular parasites (Hamano et al., 2003; Rosas et al., 2006), helminth infection (Artis et al., 2004), bacterial infection (Pearl et al., 2004) (Holscher et al., 2005), and numerous autoimmune models (Amadi-Obi et al., 2007; Batten et al., 2006; Fitzgerald et al., 2007; Stumhofer and Hunter, 2008; Troy et al., 2009; Villarino et al., 2008).

The role of Ahr and Lipoxin in controlling immunopathology

Another factor that contributes to the control of chronic toxoplasmosis is Lipoxin A4 (LXA4), a product of the reaction catalyzed by the enzyme 5-lipoxygenase (5LO). Mice deficient in 5LO succumb to toxoplasmosis approximately one month post infection, having reduced parasite burdens relative to wild type controls (Aliberti et al., 2002b). 5LO-deficiency is associated with increased infiltration of inflammatory cells into the brain, and elevated production of the cytokines IL-12, IFN-γ, and TNF-α. Expression of LXA4 is induced by infection with *T. gondii* and administration of an LXA4 analogue can rescue 5LO-deficient mice, implicating this molecule as a negative regulator of inflammation. One known mechanism by which LXA4 can inhibit immune responses is by serving as a ligand for the aryl hydrocarbon receptor (Ahr) (Schaldach et al., 1999). Ahr is an intracellular signaling molecule that translocates to the nucleus upon binding to its ligand (Abel and Haarmann-Stemmann, 2010). Ligation of Ahr by LXA4 inhibits the production of IL-12 from DCs in a manner dependent upon the signaling molecule Suppresser of Cytokine Signaling 2 (SOCS2), which is also required for survival during toxoplasmosis (Aliberti et al., 2002a; Machado et al., 2006). Consistent with this mechanism as a means to control the immune response during toxoplasmic encephalitis, mice deficient in Ahr have reduced parasite burdens, but succumb to chronic infection with *T. gondii* (Sanchez et al., 2010).

Other factors involved in controlling immune pathology

TLR11 is another factor necessary to control the immune response during acute toxoplasmosis (Yarovinsky et al., 2008). In the absence of TLR11, mice develop fat necrosis and pancreatic inflammation, which does not correlate with increased parasite burden in the pancreas. This inflammation is mediated by IL-12, IFN-γ and NK cells, but is independent of T lymphocytes. The cytokines IL-18 and IL-1β are also partially responsible for the development of pancreatic inflammation, as neutralization of either of these cytokines decreases inflammation. These results raise the question as to what anti-inflammatory mechanisms may be initiated by TLR11 ligation. Given the prominent role of IL-12 in eliciting production of IL-10 from NK cells (Perona-Wright et al., 2009), it may be that decreased IL-12 levels in the absence of TLR11 result in insufficient amounts of IL-10 to prevent pancreatic pathology, although additional experiments are required to test this model.

Another pathway involved in protection of mice against severe immunopathology during acute toxoplasmosis involves the cleavage of fibrinogen to produce fibrin, as part of the cascade responsible for blood clotting (Furie and Furie, 1988). Fibrin levels are increased upon infection with *T. gondii* in a TNF-α dependent manner, while IFN-γ negatively regulates these events (Mullarky et al., 2006). Mice lacking fibrin exhibit normal control of parasite burden and intact immune responses, but succumb to infection within the first 15 days, associated with an IFN-γ dependent liver pathology characterized by necrosis, hemorrhaging, and diffuse inflammatory infiltrates (Johnson et al., 2003). Thus, fibrin prevents hemorrhaging and severe pathology during toxoplasmosis.

Summary

While this chapter has summarized much of the current knowledge of toxoplasmosis, it has also highlighted areas of ambiguity in the literature. Although evidence suggests that CD8α dendritic cells are critical for generating adaptive immune responses, the roles of myeloid dendritic cells and plasmacytoid dendritic cells in this process are less clear. Furthermore, although an infection-induced increases in numbers of dendritic cells have been observed, it is unclear what factors

regulate dendritic cell homeostasis during infection. The specific types of interactions between parasites and host cells (e.g. active inavasion or phagocytosis) that lead to antigen presentation are also unknown. Chapter 2 of this thesis will explore the role of Flt3L, a cytokine known to be involved in dendritic cell homeostasis, in immunity to *T. gondii*. In Chapter 3, the specific interactions between parasites and host cells necessary for the generation of adaptive immunity will be explored. Lastly, the findings from these chapters will be discusses in the context of the current understanding of toxoplasmosis, their potential applications to medicine, and future directions for research in this field.

Cytokines	Sources	Phenotype of knockout	Functions
• IL-12	• Dendritic cells (Hou et al., 2011; Liu et al., 2006; Mashayekhi et al., 2011) • Neutrophils (Bliss et al., 1999a; Bliss et al., 1999b; Whitmarsh et al., 2011) • Inflammatory monocytes (Mordue and Sibley, 2003; Whitmarsh et al., 2011) • Macrophages (Gazzinelli et al., 1996b; Mordue and Sibley, 2003; Whitmarsh et al., 2011)	• Succumb within 10 days of infection (Gazzinelli et al., 1994; Yap et al., 2000) • Inability to control parasite burden (Gazzinelli et al., 1994; Yap et al., 2000)	• Promotes T cell proliferation and differentiation (Gazzinelli et al., 1994; Wilson et al., 2008) • Promotes Natural Killer cell responses (Gazzinelli et al., 1993b; Hunter et al., 1994) • Promotes IFN-γ production (Gazzinelli et al., 1993b; Gazzinelli et al., 1994) • Promotes IL-10 expression from natural killer cells (Perona-Wright et al., 2009)
• IFN-γ	• Natural killer cells (Denkers et al., 1993; Hunter et al., 1994; Johnson et al., 1993; Sher et al., 1993) • $CD4^+$ T cells (Gazzinelli et al., 1992a) • $CD8^+$ T cells (Gazzinelli et al., 1992a)	• Succumb within 10 days of infection (Suzuki et al., 1988) • Inability to control parasite burden (Suzuki et al., 1988)	• Promotes iNOS expression (Adams et al., 1990) • Promotes p47 GTPase-mediated killing of *T. gondii* (Howard et al., 2011; Taylor et al., 2000) • Promotes Tryptophan degradation (Daubener et al., 1996; Daubener et al., 2001; Murray et al., 1989; Pfefferkorn, 1984)

• TNF-α	• Neutrophils (Bennouna et al., 2003; Bliss et al., 1999a) • Dendritic cells (Bennouna et al., 2003) • Macrophages (Li et al., 1994) • Microglia (Schluter et al., 2001) • T cells (Schluter et al., 1997)	• Succumb ~3-4 weeks post-infection (Deckert-Schluter et al., 1998; Schluter et al., 2003; Yap et al., 1998) • Inability to control parasite burden (Deckert-Schluter et al., 1998; Schluter et al., 2003; Yap et al., 1998)	• Promotes macrophage activation (Chang et al., 1990) • Promotes control of parasite in non-hematopoietic cells (Yap and Sher, 1999) • Promotes iNOS expression (Deckert-Schluter et al., 1998; Gazzinelli et al., 1993a; Langermans et al., 1992; Schluter et al., 2003)
• IL-6	• Monocytes (Pelloux et al., 1994) • Astroglia (Fischer et al., 1997) • Stromal cells (Chou et al., 2012) • Retinal pigment epithelial cells (Nagineni et al., 2000)	• Increased susceptibility 2-4 weeks post-infection (Jebbari et al., 1998) • Increased parasite burden (Jebbari et al., 1998; Suzuki et al., 1997)	• Necessary for optimal neutrophil responses (Jebbari et al., 1998) • Necessary for optimal IFN-γ responses (Jebbari et al., 1998; Suzuki et al., 1997)
• LT- α	• Lymphocytes (Browning et al., 1997)	• Succumb 2-4 weeks post-infection (Schluter et al., 2003)	• Necessary for normal secondary lymphoid architecture (De Togni et al., 1994) • Necessary for optimal antibody and IFN-γ responses early during infection (Schluter et al., 2003) • Necessary for optimal expression of iNOS (Deckert-Schluter et al., 1998)

• IL-10	• Natural killer cells (Perona-Wright et al., 2009) • Macrophages (Deckert-Schluter et al., 1997) • $CD4^+$ T cells (Jankovic et al., 2007) • $CD8^+$ T cells (Deckert-Schluter et al., 1997)	• Succumb 1-2 weeks post-infection (Gazzinelli et al., 1996b) • Severe immunopathology (Gazzinelli et al., 1996b)	• Inhibits CD4+ T cell-mediated pathology (Gazzinelli et al., 1996b)
• IL-27	• Antigen Presenting Cells (Pflanz et al., 2002)	• Succumb within 15 days post-infection (Villarino et al., 2003) • Severe immunopathology (Villarino et al., 2003)	• Inhibits IL-17 production (Stumhofer et al., 2006) • Inhibits IL-2 production (Villarino et al., 2003) • Promotes IL-10 production (Stumhofer et al., 2007) • Promotes PD-L1 expression (Hirahara et al., in press, 2012)
• CD40L (Surface protein)	• Expressed on T cells (Banchereau et al., 1994)	• Succumb 30-80 days post-infection (Reichmann et al., 2000) • Inability to control parasite burden (Reichmann et al., 2000)	• Promotes Th1 responses in humans (Subauste et al., 1999) • Promotes iNOS expression (Jana et al., 2001) • Promotes xenophagic killing of *T. gondii* (Portillo et al., 2010)

Table 1.1. Cytokines involved in immunity to *T. gondii.*

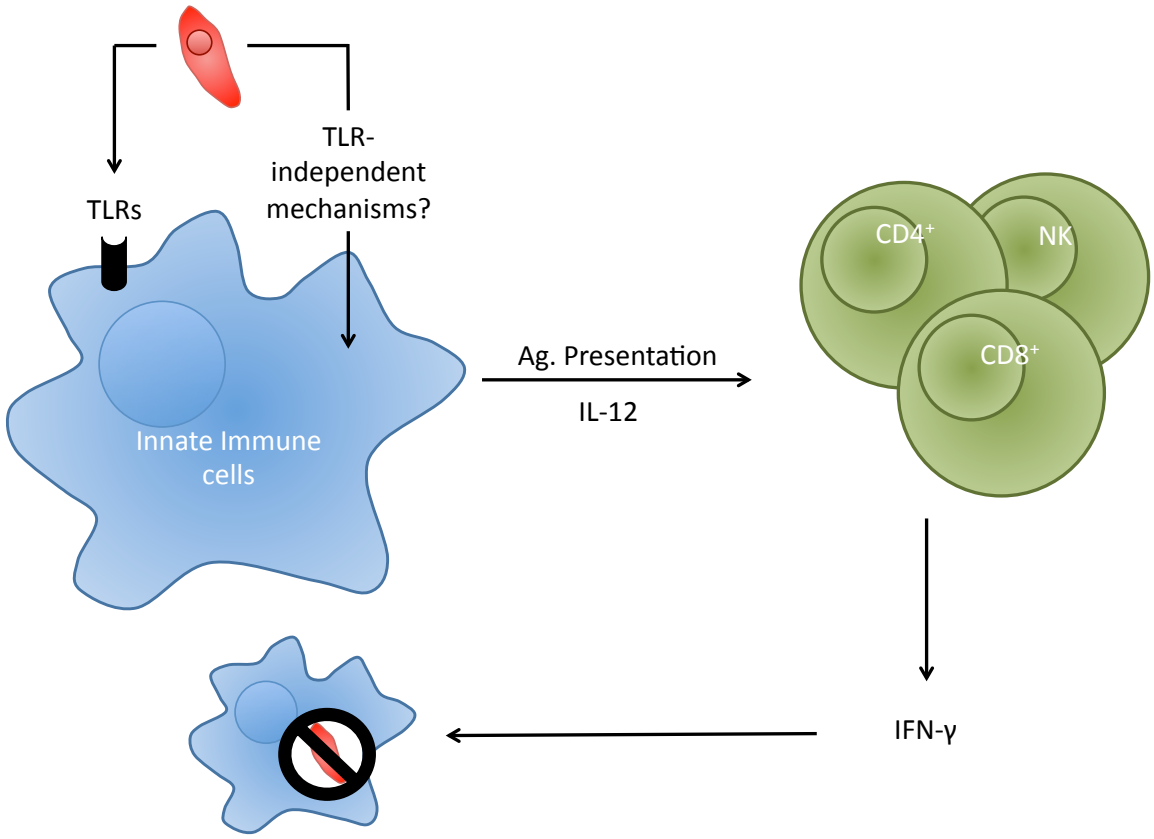

Figure 1.1: Immune response to *T. gondii*. During toxoplasmosis, TLRs 2,4, 11 and 12 sense *T. gondii*, and other unidentified TLR-independent mechanisms are likely involved as well. In response to the parasite, innate immune cells such as dendritic cells produce IL-12 and present antigen to $CD4^+$ and $CD8^+$ T cells. IL-12 drives the production of IFN-γ from $CD4^+$ T cells, CD8+ T cells and NK cells. IFN-γ mediates anti-parasitic effector mechanisms in infected host cells.

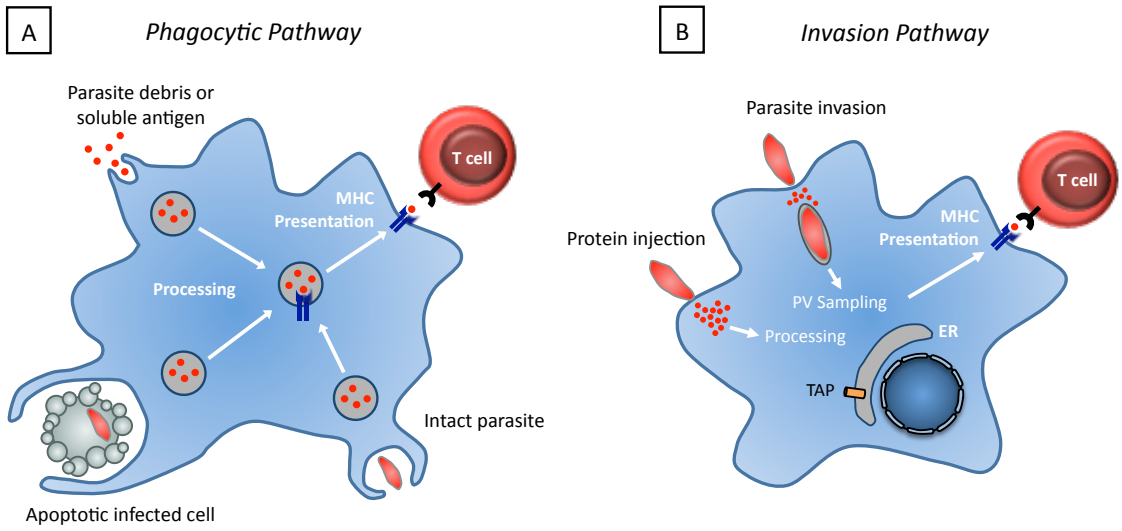

Figure 1.2: Potential antigen presentation pathways. A. Antigen may be acquired through the phagocytosis of infected cells, intact parasites, parasite antigens, or through the endocytosis of parasite debris. **B.** Antigen may also be acquired by infected cells through the release of soluble antigens from the parasite, or through the sampling of antigens from the parasitophorous vacuole, which may be mediated by fusion of the parasitophorous vacuole with the endoplasmic reticulum. Antigens may also be injected into the host cell through abortive invasion events.

CHAPTER 2: DEFICIENCY IN FLT3L LEADS TO ACUTE SUSCEPTIBILITY TO TOXOPLASMOSIS

Abstract

Toxoplasma gondii is an intracellular protozoan parasite and a prominent cause of disease in patients with inherited or acquired deficiencies in T cells. Dendritic cells (DCs) are critical for immunity to this parasite, however little is known about the factors that regulate DC homeostasis during infection. The cytokine fms-like tyrosine kinase-3 ligand (Flt3L) is a growth factor that promotes dendritic cell development, and challenge of mice with *T. gondii* is associated with increased numbers of DCs that express the receptor for Flt3L (Flt3). When mice deficient in Flt3L (Flt3L KO mice), which have basal defects in numbers of DCs, were infected with an avirulent strain of *T. gondii*, they displayed increased growth of parasites and susceptibility to acute infection. This phenotype correlated with decreased production of the cytokines IL-12 and interferon-gamma (IFN-γ), and impaired natural killer cell responses. Surprisingly, infection of Flt3L KO mice induced increased numbers of DCs, and expansion of parasite-specific $CD4^+$ and $CD8^+$ T cells, however these T cells were impaired in their ability to produce IFN-γ. Finally, despite marked defects in the numbers of DCs in the Flt3L KO mice, the administration of exogenous IL-12 alone was sufficient to prevent acute susceptibility. Together, these results highlight the importance of the Flt3L pathway for the development of DCs required for resistance to toxoplasmosis, but demonstrate that there are Flt3L-independent pathways that are sufficient to prime $CD4^+$ and $CD8^+$ T cells capable of controlling acute toxoplasmosis.

Introduction

Toxoplasma gondii is an intracellular protozoan parasite of medical and veterinary importance (Dubey, 2008). Successful control of this infection requires a Th1 immune response, characterized by the production of the cytokine IL-12 from innate myeloid cells such as dendritic cells (DCs), which drives the production of interferon-gamma [IFN-γ] from natural killer (NK) cells, $CD4^+$ T cells and $CD8^+$ T cells (Gazzinelli et al., 1992a; Gazzinelli et al., 1994; Suzuki et al., 1988). IFN-γ, in combination with other inflammatory signals, acts directly on infected host cells to induce anti-microbial effector mechanisms that can kill the parasite or limit its ability to replicate (Dupont et al., 2012). Although NK cells serve as a source of IFN-γ during acute infection, production of IFN-γ from $CD4^+$ and $CD8^+$ T cells is required for long-term protection (Gazzinelli et al., 1993b; Gazzinelli et al., 1994; Hunter et al., 1994).

With the recognition that IL-12 was critical for innate and adaptive immunity to *T. gondii*, there has been an interest in defining the cellular sources of this cytokine and understanding how these cells sense the parasite. To date, Toll-like receptors (TLR) 2,4,11, and 12, as well as the chemokine receptor CCR5 have all been proposed to directly sense parasite antigens (Aliberti et al., 2000; Debierre-Grockiego et al., 2007; Koblansky et al., 2013; Yarovinsky et al., 2005). Additionally, multiple cellular sources of IL-12 during toxoplasmosis have been identified that include neutrophils (Egan et al., 2008), inflammatory monocytes (Goldszmid et al., 2012), macrophages (Gazzinelli et al., 1996b), plasmacytoid DCs (Koblansky et al., 2013; Pepper et al., 2008) and $CD8\alpha^+$ DCs (Mashayekhi et al., 2011; Reis e Sousa et al., 1997). Regardless, there is strong evidence that DCs are a critical source of IL-12, as mice in which DCs are selectively depleted or those in which DCs lack the TLR signaling molecule MyD88 have reduced IL-12 production and are acutely susceptible to toxoplasmosis (Hou et al., 2011; Scanga et al., 2002). Other studies have shown that the administration of Soluble *T. gondii* Antigen (STAg) induces IL-12 production predominantly by CD8α DCs (Reis e Sousa et al., 1997). Furthermore, Batf3 KO mice, which lack CD8α DCs, infected with *T. gondii* display impaired IL-12 production and increased susceptibility to this challenge (Mashayekhi et al., 2011). In contrast to these studies, a

recent report concluded that CD11b^{HI} DCs derived from inflammatory monocytes were the major source of IL-12 in vivo (Goldszmid et al., 2012).

While there is strong evidence that DCs have an important role in resistance to *T. gondii,* little is known about the host factors that influence their homeostasis during this infection. Multiple reports have established that in mice challenged with *T. gondii*, there are increased numbers of plasmacytoid DCs in lymphoid tissues, accumulation of CD11b^{HI} DCs at the site of infection and in the draining lymph nodes, and expansion of CD8α DCs in the spleen (Mashayekhi et al., 2011; Pepper et al., 2008; Tait et al., 2010). These changes may result from increased migration of DCs to these sites, the development of inflammatory monocytes into DCs, increased proliferation or survival of DCs, or increased differentiation and proliferation of DC precursor cells (Liu and Nussenzweig, 2010). Indeed, there is evidence that monocytes develop into CD11b^{HI} DCs during toxoplasmosis (Goldszmid et al., 2012), and recent work has identified a Batf3-independent pathway by which inflammation can induce the development of CD8α DCs (Tussiwand et al., 2012).

One of the key cytokines that regulates DC development and homeostasis is Fms-like tyrosine kinase-3 ligand (Flt3L), which promotes the differentiation and proliferation of dendritic cell precursors, as well as the expansion of mature DCs (Watowich and Liu, 2010). Flt3L can exist in a soluble or transmembrane form, and is expressed by bone marrow stromal cells and mast cells, as well as numerous other cell types whereas its receptor, Flt3, is expressed on dendritic cell precursors and mature DCs (Bertho et al., 2008; Lim et al., 2012; Lyman and Jacobsen, 1998). While Flt3L has been most prominently linked to DC development, there is evidence that it also impacts the function of DCs. Thus, Flt3L has been proposed to extend the period of antigen presentation by promoting the proliferation of antigen-presenting DCs (Liu et al., 2007), and has been shown to promote dendritic cell-mediated activation of NK cells (Eidenschenk et al., 2010).

In order to better understand the factors that influence DC homeostasis during infection, studies were performed to assess the role of Flt3L-dependent DC populations in resistance to *T. gondi.* Infection with *T. gondii* induced the expansion of Flt3^{+ve} DCs, and mice deficient in Flt3L

(Flt3L KO mice) were unable to control early parasite burden. This phenotype correlated with decreased production of IL-12 and IFN-γ, reduced numbers of IL-12-producing DCs at the site of infection, and impaired NK cell responses. Surprisingly, infection of Flt3L KO mice did result in a significant increase in DCs, associated with the expansion of parasite-specific $CD4^+$ and $CD8^+$ T cell populations, however the ability of these T cells to produce IFN-γ was impaired. Lastly, Flt3L KO mice could be rescued by the administration of exogenous IL-12, thus highlighting the role of DCs as a source of IL-12 during toxoplasmosis, and demonstrating that DC expansion and priming of naïve T cells can occur independently of Flt3L.

Materials and Methods

Ethics Statement All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Animal Welfare Assurance Reference Number #A3079-01) and were in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

Mice Flt3L KO and C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). All mice were kept in specific-pathogen-free conditions at the School of Veterinary Medicine at the University of Pennsylvania.

Infections All infections were performed with 10^3 Pru-OVA parasites or Pru-OVA-Tomato parasites, which have been previously described (John et al., 2009; Pepper et al., 2004). Parasites were cultured and maintained by serial passage on human foreskin fibroblast cells in the presence of parasite culture media [71.7% DMEM (Corning), 17.9% Medium 199 (Invitrogen), 9.9% Fetal Bovine Serum (FBS)(Invitrogen), 0.45% Penicillin and Streptomycin (Invitrogen)(final concentration of 0.05 units/ml Penicillin and 50 µg/ml Streptomycin), 0.04% Gentamycin (Invitrogen)(final concentration of 0.02 mg/ml Gentamycin)]. For infections, parasites were harvested and serially passaged through 18, 20 and 26 gauge needles (BD) before filtration with a 5 µM filter (Sartorius Stedim). Parasites were washed extensively with PBS and mice were injected intraperitoneally with 10^3 parasites suspended in PBS. IL-12 was obtained from PeproTech.

Cell culture and tissue harvesting Peritoneal exudate cells (PECS) were obtained by peritoneal lavage with 5 ml of PBS. Splenocytes were obtained by grinding spleens over a 40 μM filter (Biologix) and washing them in complete media. Red blood cells were then lysed by incubating for 5 minutes at room temperature in 5 ml of lysis buffer [0.864% ammonium chloride (Sigma-Aldrich) diluted in sterile de-ionized H_2O)], followed by washing with complete media. Bone marrow-derived macrophages were obtained using previously described methods (Robben et al., 2004; Whitmarsh et al., 2011). For experiments in which cells were restimulated to measure cytokine levels, cells were incubated in complete media in the presence of Brefeldin A (0.01 mg/ml), with or without PMA (0.1 μg/ml) and ionomycin (0.1 μg/ml) for four hours.

Flow Cytometry Cells were washed with FACS Buffer [1x PBS, 0.2% bovine serum antigen (Sigma), 1 mM EDTA (Invitrogen)], stained with LIVE/DEAD® Fixable Aqua Dead Cell marker (Invitrogen) and incubated in Fc block [99.5% FACS Buffer, 0.5% normal rat serum (Invitrogen), 1 μg/ml 2.4G2 (BD)] prior to staining. APC-MHCI-SIINFEKL Tetramer was obtained from Beckman-Coulter. PE-MHCI-SVLAFRRL was obtained as a generous gift from the NIH Tetramer Core Facility. The following antibodies were used for staining: Ki67 Alexa Fluor®488 (BD, B56), CD3 APC-eFluor®780 (eBioscience, 17A2), CD8 eFluor®450 (eBioscience, 53-6.7), CD11a PerCP-Cy5.5 (Biolegend, H155-78), MHCII PE (eBioscience, M5/114.15.2), NK1.1 PE (BD, PK136), CD19 PE (eBioscience, 1D3), Foxp3 eFluor®450 (eBioscience, FJK-16a), CD4 Pe-Cy7 (eBioscience, GK1.5), CD3 FITC (BD, 145-2C11), NK1.1 FITC (eBioscience, PK136), CD19 FITC (eBioscience, 1D3), Gr-1 PerCP-Cy5.5 (eBioscience, RB6-8C5), CD11c PE-Cy7 (eBioscience, N418), CD11b APC-eFluor®780 (eBioscience, M1/70), MHCII AF700 (Biolegend, M5/114.15.2), MHCII Pacific Blue (BioLegend, M5/114.15.2), PE Flt3 (eBioscience, A2F10), eFluor®660 Ly6g (eBioscience, 1A8), Alexa Fluor® IL-12p40 (eBioscience, C17.8), FITC DX5 (BD, DX5), eFluor®660 NKp46 (eBioscience, 29A1.4), Alexa Fluor®647 T-bet (BioLegend, 4B10), Pe-Cy7 IFN-γ (eBioscience, XMG1.2). Intracellular staining for transcription factors was performed using the Foxp3/ Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer's instructions. Intracellular cytokine staining was performed by fixing cells in 2% paraformaldehyde,

followed by permeabalization and staining in 0.5% saponin (Sigma-Aldrich). Samples were run on a FACSCanto (BD) or LSR Fortessa (BD) and analyzed using FlowJo Software (TreeStar).

ELISAs IL-12p40 was measured by ELISA using C17.8 and biotinylated C15.6 antibodies. IFN-γ was measured by ELISA using AN18 and biotinylated R4-6A2 (eBioscience) antibodies. Flt3L was measured using the Mouse/Rat Flt-3 Ligand Quantikine ELISA Kit.

Statistical Analysis Statistical analysis was performed using PRISM software (Graphpad Software). Significance was calculated using an unpaired two-tailed student's t-test except when otherwise noted. *p<0.05; **p<0.005; ***p<0.0005.

Results

Infection induced changes in Flt3-expressing dendritic cells

Given the marked changes in DC populations observed during toxoplasmosis (Mashayekhi et al., 2011; Pepper et al., 2008; Tait et al., 2010), and the known role of Flt3L in DC homeostasis (Liu and Nussenzweig, 2010), experiments were performed to assess how infection with *T. gondii* affected expression of Flt3L and Flt3. When C57Bl/6 mice were challenged using the Pru strain of *T. gondii* and examined at day 5 post-infection, Flt3L was detectable in the sera of these mice at levels similar to those in naïve mice (Figure 2.1a). Expression of Flt3 could be detected on peritoneal DCs from naïve and infected mice, but not on macrophages, neutrophils or monocytes (Figure 2.1b). Two distinct populations of DCs could be distinguished on the basis of Flt3 expression ($Flt3^{LOW}$ and $Flt3^{HI}$), and consistent with previous reports (Ginhoux et al., 2009), $Flt3^{LOW}$ cells displayed high levels of CD11b, whereas $Flt3^{HI}$ cells displayed relatively lower levels of CD11b (Figure 2.1c). Infection induced no changes in the level of Flt3 expression on either the $Flt3^{LOW}$ or $Flt3^{HI}$ DC populations (Figure 2.1d). Although no significant changes in numbers of $Flt3^{LOW}$ DCs were apparent, infection induced a significant increase in numbers of $Flt3^{HI}$ DCs at the site of infection (Figure 2.1e). Similar results were apparent when Flt3 expression on splenic DCs was examined (data not shown).

Flt3L KO mice are unable to control infection with *T. gondii*

When wild type (WT) and Flt3L KO mice were infected intraperitoneally with the Pru strain of *T. gondii*, all WT mice survived challenge, whereas the majority of Flt3L KO mice succumbed to infection within 12 days (Figure 2.2a). To understand the basis of this phenotype, Flt3L KO and wild-type mice were infected and sacrificed at day 8 post-infection to assess parasite burden and pathology. At this time point, Flt3L KO mice demonstrated a major increase in the parasite burden based on the percentage of infected cells, as determined by examining cells obtained from the peritoneal cavity by microscopy, and by using fluorescently-labeled parasites to measure parasite burden in the spleen (Figure 2.2b). Elevated parasite burdens were detected in the livers and lungs of Flt3L KO mice (data not shown). Histological examination of these tissues in the WT and Flt3L KO typically revealed areas of inflammation associated with the presence of parasites, but these were more severe in the Flt3L KO mice. However, in the liver there were also sites of extra-medullary hematopoiesis that were prominent in the WT mice – but these were markedly reduced in the Flt3L KO mice (Figure 2.2c). Collectively, these results establish that Flt3L KO mice are unable to control parasite burden.

Impaired dendritic cell responses in Flt3L KO mice

To assess the cause of the increased susceptibility of Flt3L KO mice to toxoplasmosis, levels of the cytokines IL-12 and IFN-γ were measured in the sera on day 8 post-infection. Deficiency in Flt3L was associated with significantly decreased levels of both IL-12 (60.0% reduction; $p<0.005$) and IFN-γ (45.7% reduction; $p<0.05$)(data not shown). To assess DC responses infected WT and KO mice were sacrificed at day 5 post-challenge, a time point when infection rates in the PECS were typically less than 0.5% in WT mice and 2% in Flt3L KO mice (data not shown). While infection with *T. gondii* was found to induce a significant increase in numbers of DCs at the site of infection in both WT and Flt3L KO mice, numbers of DCs were reduced in infected Flt3L KO mice, relative to WT controls (Figure 2.3a). In contrast, infection-induced increases in neutrophils, monocytes and macrophages were intact in Flt3L KO mice (data not shown). Examination of IL-12 production by intracellular cytokine staining revealed that neutrophils ($CD3^-$

^{ve}CD19^{-ve}NK1.1^{-ve}CD11c^{LOW}Ly6g^{+ve}) and macrophages (CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{LOW}Ly6g^{-ve}CD11b^{INT-HI}) were minor sources of IL-12 at the local site of infection (data not shown) and that DCs (CD3^{-ve}CD19-^{ve}NK1.1^{-ve}CD11c^{HI}MHCII^{HI}) were the major sources of IL-12 in WT mice (Figure 2.3b). Nevertheless, Flt3L KO mice were found to produce less IL-12 on a per-cell basis, resulting in a significant reduction in the number of total IL-12-producing cells in the peritoneal cavities of Flt3L KO mice (Figure 2.3c). IL-12-producing DCs in WT mice were negative for CD8α (very few CD8α^{+} DCs could be isolated from the peritoneal cavity) and tended to display low levels of CD11b (Figure 2.3d). Due to the scarce nature of IL-12^{+} DCs in the peritoneal exudate cells (PECS) of Flt3L KO mice, these cells could not be phenotypically examined. Numbers of IL-12-producing DCs were also reduced in the spleens of infected Flt3L KO mice when compared to WT mice (data not shown).

In addition to the infection-induced increase in dendritic cell number apparent in the peritoneal cavity at day 5, an increase in numbers of DCs was also apparent in the spleens of WT and FLt3L KO mice at day 8 post infection, but not at any earlier time points examined (Figure 2.4a, data not shown). Despite this, total numbers of splenic DCs were reduced in both naïve and infected Flt3L KO mice, relative to WT controls. Numbers of DCs belonging to each individual subset examined (CD11b^{HI} DCs, CD8α^{+} DCs, CD11b^{LOW} CD8α^{-ve} DCs) increased in both infected Flt3L KO and infected WT mice (relative to naïve controls), although the composition of the total dendritic cell population varied between WT and Flt3L KO mice, as a larger percentage of DCs were CD11b^{HI} in Flt3L KO mice than in WT mice (Figure 2.4a,b). These results demonstrate that the infection-induced increase in numbers of splenic DCs can occur independently of Flt3L, and suggest that the underlying defect in DC numbers in Flt3L KO mice contributes to the overall defect in IL-12 production.

Impaired NK cell responses in Flt3L KO mice

Since IL-12 promotes NK cell production of IFN-γ during toxoplasmosis, the NK cell response in WT and Flt3L KO mice was compared to determine if defects in this population contribute to the systemic defect in IFN-γ present in infected Flt3L KO mice. Similar to previous reports (Guimond

et al., 2010; McKenna et al., 2000), naïve Flt3L KO mice exhibited decreased numbers of NK cells relative to wild type controls (Figure 2.5a). This defect was not restored by infection, as numbers of NK cells remained lower than wild type controls at day 5 post-infection. NK cells were also less activated in Flt3L KO mice, as demonstrated by their failure to upregulate expression KLRG1, the transcription factor T-bet and the production of the cytokine IFN-γ, all markers of effector function (Figure 2.5b). Collectively, these results suggest that NK cell responses are impaired in Flt3L KO mice.

Analysis of $CD4^+$ and $CD8^+$ T cell responses

Because DCs and their production of IL-12 are required for the induction of $CD4^+$ and $CD8^+$ T cell responses and long term resistance to toxoplasmosis, the $CD4^+$ and $CD8^+$ T cell responses to *T. gondii* in Flt3L KO mice were assessed. To identify polyclonal $CD4^+$ and $CD8^+$ T cell responses the combination of the activation marker CD11a and the marker of cellular proliferation Ki67 were utilized (McDermott and Varga, 2011; Rai et al., 2009; Scholzen and Gerdes, 2000). Additionally, because mice were infected with a strain of *T. gondii* engineered to secrete Ovalbumin (OVA), CD11a was combined with MHCI tetramers that bind to $CD8^+$ T cells specific for the *T. gondii* epitope Tgd_057, and MHCI tetramers that bind to $CD8^+$ T cells specific for OVA. Although at day 8 post-infection, decreased percentages and numbers of polyclonal activated $CD4^+$ and $CD8^+$ T cells were apparent in the spleens of Flt3L KO mice, expansion of $CD4^+$ and $CD8^+$ T cells did occur in Flt3L KO mice (Figure 2.6a,b). Similarly, a defect in numbers of OVA-specific $CD8^+$ T cells was apparent, although infection induced an increase in OVA-specific $CD8^+$ T cells in Flt3L KO mice (data not shown). Since production of IFN-γ from $CD4^+$ and $CD8^+$ T cells is critical for immunity to *T. gondii*, IFN-γ production from $CD4^+$ and $CD8^+$ T cells was examined. Both $CD4^+$ and $CD8^+$ T cells from Flt3L KO mice displayed impaired production of IFN-γ directly ex vivo (Figure 2.6c,d). Stimulation of T cells with PMA and ionomycin also resulted in less IFN-γ production from $CD4^+$ and $CD8^+$ T cells from Flt3L KO mice than was observed from wild type controls. These results suggest that despite impaired numbers of DCs and decreased IL-12 production, sufficient antigen presentation occurs during toxplasmosis in Flt3L KO mice to induce

T cell expansion, however deficient production of IFN-γ from $CD4^+$ and $CD8^+$ T cells likely contributes to the inability to control parasite burden.

Administration of exogenous IL-12 can rescue Flt3L KO mice

Since susceptibility to toxoplasmosis in Flt3L KO mice was associated with decreased IL-12 production, and because IL-12 is critical for survival during toxoplasmosis, IL-12 was administered to Flt3L KO mice to determine the effect this has on parasite burden. Administration of exogenous IL-12 resulted in a dramatic decrease in parasite burden in Flt3L KO mice (Figure 2.7a). Although treatment with IL-12 did not increase IFN-γ production from NK cells on a per-cell basis (Figure 2.7b), IL-12 treatment significantly increased the numbers of natural killer cells at the site of infection in Flt3L KO mice (Figure 2.7c). This finding highlights the role of DCs as critical producers of IL-12 during toxoplasmosis.

Discussion

Previous studies have observed an infection-induced expansion of DCs during toxoplasmosis, and identified DCs as major sources of IL-12 critical for controlling infection (Goldszmid et al., 2009; Hou et al., 2011; Mashayekhi et al., 2011; Pepper et al., 2008; Scanga et al., 2002; Tait et al., 2010). However, questions remained regarding the underlying mechanisms that lead to the expansion of DCs, and the Flt3/Flt3L interaction represented one candidate that might contribute to DC expansion and homeostasis during this infection. Herein, mice deficient in Flt3L were found to be acutely susceptible to infection with *T. gondii*. Infection with *T. gondii* resulted in an increase in numbers of DCs in the spleen and at the site of infection in both WT and Flt3L KO mice, and the DCs from the PECS of Flt3L KO mice displayed defective IL-12 production on a per-cell basis. This defect could potentially result from a role for Flt3L in promoting IL-12 production from DCs, as Flt3L has previously been shown to promote IL-12 production (Eidenschenk et al., 2010; Vollstedt et al., 2004). Alternatively, this lack of IL-12 could be a consequence of the defective production of IFN-γ from NK cells, which is necessary for optimal production of IL-12 at the site of infection (Goldszmid et al., 2012). In addition to the impaired production of IL-12 on a per-cell basis, Flt3L KO mice also have reduced numbers of

DCs, which likely contributes to the decreased systemic levels of IL-12 observed during toxoplasmosis in these mice. The administration of exogenous IL-12 resulted in decreased parasite burdens in Flt3L KO mice, thus highlighting the critical role of DCs as sources of IL-12, and pointing toward defective IL-12 production as the likely underlying cause of susceptibility in Flt3L KO mice.

The finding that $CD4^+$ and $CD8^+$ T cells expanded in response to infection in Flt3L KO mice was somewhat surprising, as previous studies have demonstrated a critical role for DCs in the induction of T cell responses (Tait et al., 2010). While the decreased production of IFN-γ may result from the defect in IL-12 levels, the expansion of $CD4^+$ and $CD8^+$ T cells indicates that Flt3L-independent populations are sufficient to present antigen. This could be mediated by the DCs that exist in the absence of Flt3L, other populations of professional antigen presenting cells (such as macrophages or B cells), or non-hematopoietic cells (Dzierszinski et al., 2007).

The defect in NK cell responses observed in these studies likely results primarily from the lack of IL-12 production, as KLRG1, T-bet and IFN-γ are all induced by IL-12 signaling (Wilson et al., 2010), and treatment with IL-12 resulted in control of infection and increased numbers of IFN-γ-producing NK cells at the site of infection. Previous studies have identified DCs as an important source of IL-15, a cytokine that promotes the survival of NK cells (Ranson et al., 2003), and decreased levels of IL-15 have been implicated as the cause of the decreased numbers of NK cells present in Flt3L KO mice (Guimond et al., 2010). However, infection of IL-15 KO mice with *T. gondii* induces an expansion of NK cells to the levels present in infected WT mice, thus deficiency in IL-15 is an unlikely explanation for the defective NK cell responses observed in Flt3L KO mice during toxoplasmosis (Lieberman et al., 2004). Flt3L has also been demonstrated to enable the DC-mediated activation of NK cells in response to MCMV, and a similar role for Flt3L during toxoplasmosis cannot be ruled out (Eidenschenk et al., 2010). It is also notable that IL-12 treatment did not completely restore IFN-γ production from NK cells in Flt3L KO mice to the levels observed in WT mice, suggesting the possibility of other IL-12 independent roles for DCs in immunity to *T. gondii*. However, these data may be confounded by differences in parasite burden between WT and IL-12-treated Flt3L KO mice.

Although several studies have observed an infection-induced increase in numbers of DCs during toxoplasmosis (Mashayekhi et al., 2011; Tait et al., 2010), the mechanisms that mediate this process remain unknown. The increased numbers of $CD11b^{HI}$ DCs observed in this study and previous reports could be accounted for by the maturation of inflammatory monocytes into mature $CD11b^{HI}$ DCs (Leon et al., 2007). Indeed, monocyte-derived DCs have been reported to develop independently of Flt3 (Karsunky et al., 2003), and this is consistent with the finding that DC numbers increase in Flt3L KO mice. However, inflammatory monocytes are not thought to give rise to $CD11b^{LOW}$ DCs or $CD8\alpha^{+}$ DCs (Dominguez and Ardavin, 2010; Leon et al., 2007), raising the question of how these populations increase in number in Flt3L KO mice. Increased proliferation of mature DCs or their precursors, as well as increased differentiation of DC precursors represent additional pathways by which DC populations might increase in size. Since all of these pathways are promoted by Flt3L (Watowich and Liu, 2010), it was not expected that DC numbers would increase in the absence of this cytokine. Granulocyte-macrophage stimulating colony factor (GM-CSF) is another cytokine that can promote dendritic cell development (Vremec et al., 1997), which may be responsible for the increase in numbers of DCs during infection. It is also possible that Flt3L and GM-CSF share redundant roles in the expansion of DC populations during infection. Additionally, a recent report demonstrated that IL-12 can drive expression of the transcription factors Batf and Batf2, which can promote the development of $CD8\alpha^{+}$ DCs during infection in Batf3-deficient mice (Tussiwand et al., 2012), and this alternative pathway for dendritic cell development may contribute to the infection-induced expansion that occurs in wild type mice as well. Identifying the mechanisms that drive this infection-induced increase could allow its physiological significance to be interrogated.

While the critical role for DCs in resistance to acute toxoplasmosis has been well established, their role during toxoplasmic encephalitis is less clear, although they have been observed in the brain during chronic infection (John et al., 2011). Many studies have focused on the critical role of DCs as sources of IL-12, and questions remain regarding the extent to which IL-12 is critical during chronic infection as well. Whereas neutralization of IL-12 has no effect during chronic infection (Gazzinelli et al., 1994), sustained treatment with exogenous IL-12 is

required during chronic infection to control toxoplasmosis in IL-12-deficient mice (Yap et al., 2000). Extending the survival of Flt3L KO mice by treating them with IL-12 early during infection may allow the role of DCs during chronic infection to be interrogated, and studies are currently underway to determine if Flt3L-independent populations are sufficient to provide long-term resistance.

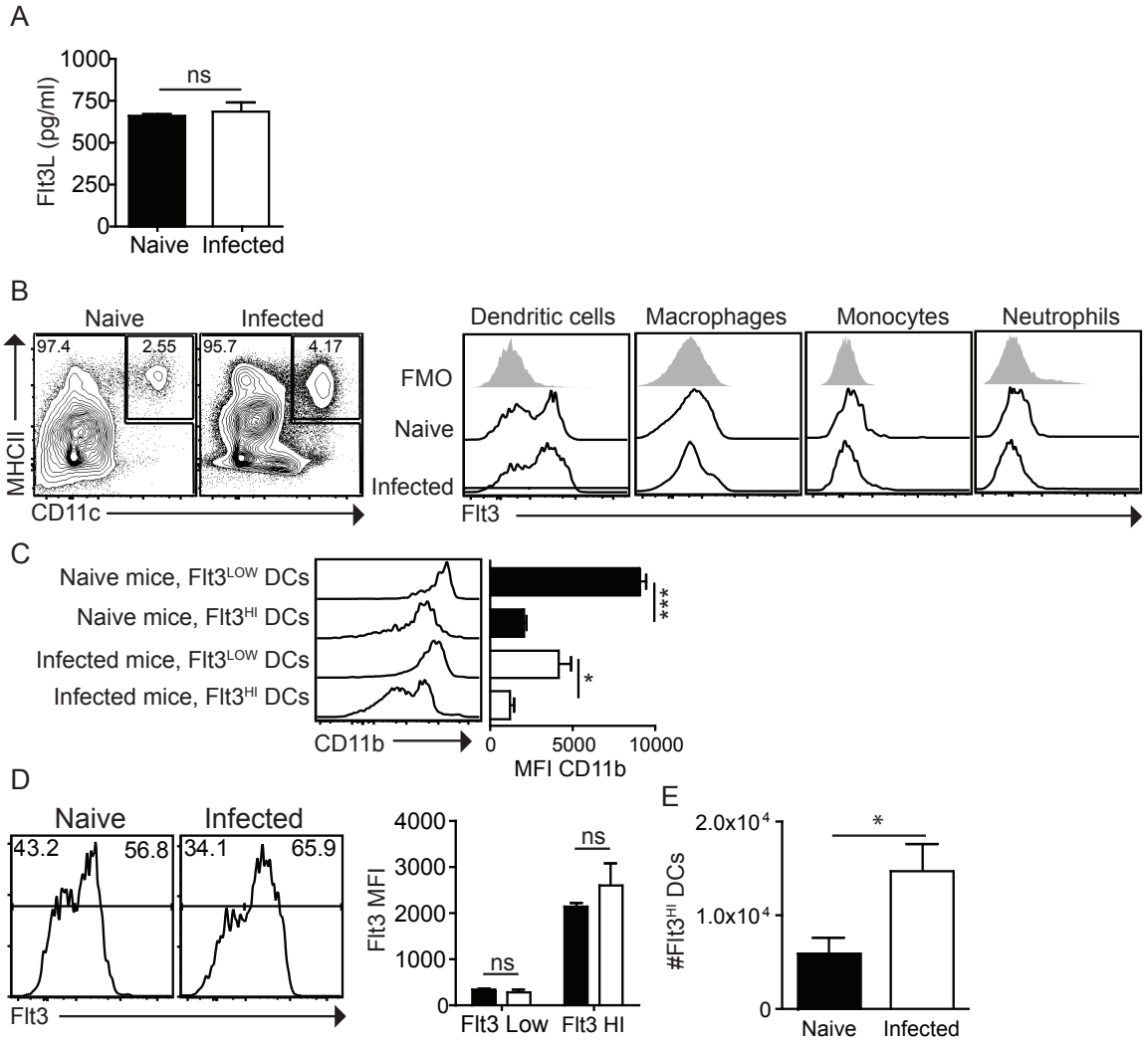

Figure 2.1. Expression of Flt3L and Flt3 during toxoplasmosis. C57BL/6 mice were infected with 10^3 tachyzoites and immune responses were analyzed 5 days post-infection. A. Levels of Flt3L from the serum of infected and naïve mice, measured by ELISA. B. Expression of Flt3 on DCs ($CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{HI}MHCII^{HI}$), macrophages ($CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{LOW-INT}Ly6c^{-ve}Ly6g^{-ve}CD11b^{HI}$), monocytes ($CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{LOW-INT}Ly6c^{+ve}$) and neutrophils ($CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{LOW-INT}Ly6g^{+ve}$) in naïve and infected mice. Flow plots on the left display the gating strategy for DCs, and the flow plots are gated on $CD3^{-ve}CD19^{-ve}NK1.1^{-ve}$ cells. C. Expression of CD11b on $Flt3^{HI}$ and $Flt3^{LOW}$ DCs. The geometric mean fluorescence intensity (MFI) of CD11b expression of the $Flt3^{HI}$ and $Flt3^{LOW}$ DC populations depicted in B are shown. D. Effects of infection on expression of Flt3. Histograms on the left

display Flt3 expression levels on peritoneal DC populations, and are gated on $CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{HI}MHCII^{HI}$ cells. Graph on the right displays the MFI of Flt3 expression on $Flt3^{HI}$ and $Flt3^{LOW}$ populations in naïve (black bars) and infected (white bars) mice. E. Numbers of $Flt3^{HI}$ DCs in the PECS of naïve and infected mice.

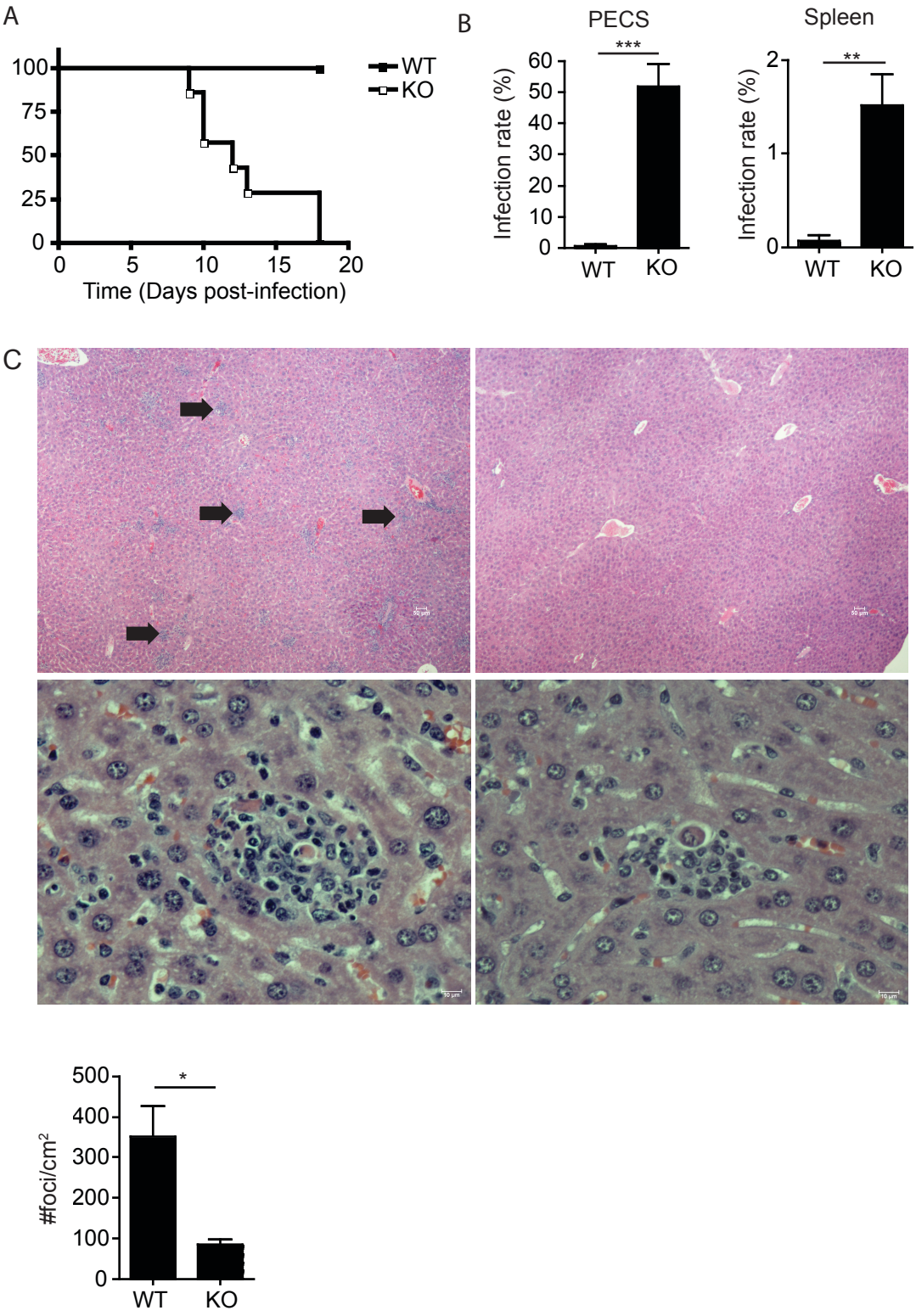

Figure 2.2. Impaired ability to control toxoplasmosis in Flt3L KO mice. A. Survival of Flt3L KO and WT mice following challenge with 10^3 tachyzoites. B. Infection rates were determined in

the peritoneal cavity (by cytospin analysis) and in the spleen (by flow cytometry) eight days following infection. C. Histological examination of liver tissue revealed numerous sites of extra-medullary hematopoiesis in infected wild-type (top left), but not Flt3L KO mice (top right). Sites of extra-medullary hematopoiesis from WT mice are shown (bottom left and right). Differences are quantified (bar graph).

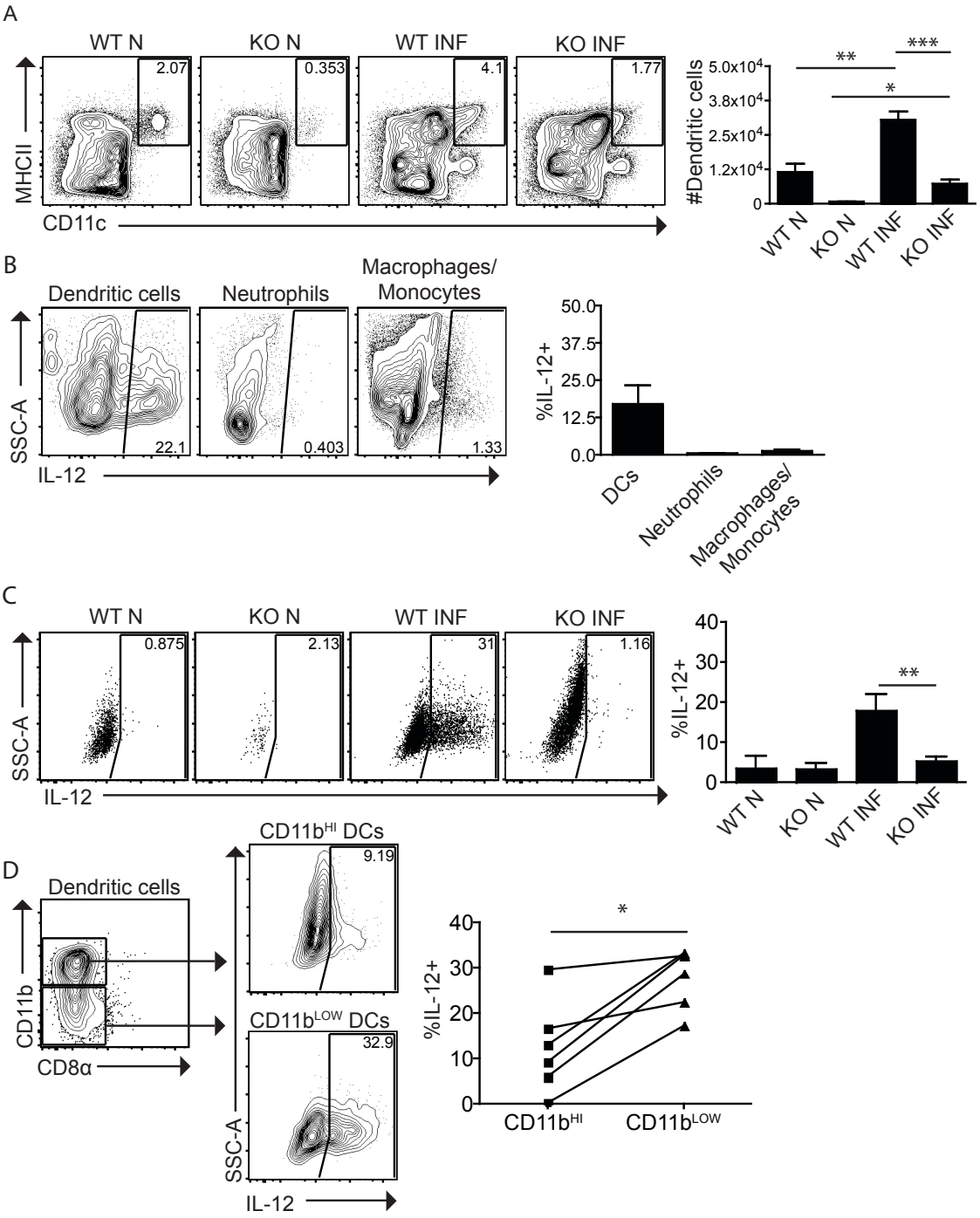

Figure 2.3: Dendritic cell responses at the site of infection in Flt3L KO mice. Mice were infected with 10^3 tachyzoites and peritoneal dendritic cell responses were analyzed 5 days post-infection. A. Numbers and percentages of DCs at the site of infection in WT and Flt3L KO mice. Flow plots are gated on $CD3^{-ve}NK1.1^{-ve}CD19^{-ve}$ cells. B. Production of IL-12p40 in WT mice by DCs ($CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{HI}MHCII^{HI}$), macrophages/monocytes ($CD3^{-ve}CD19^{-ve}NK1.1^{-}$

^{ve}CD11c$^{LOW-INT}$Ly6g^{-ve}CD11b^{INT-HI}), neutrophils (CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c$^{LOW-INT}$Ly6g^{+ve}). IL-12p40 production was measured by incubating PECS in the presence of BFA for 6 hours and staining for IL-12p40 by ICS. C. Production of IL-12p40 from peritoneal DCs of infected WT and Flt3L KO mice. IL-12 production was assayed as in B. D. IL-12 production from various DC subsets in the PECS of WT mice at day 5 post-infection. Gating strategy for CD11b^{HI} and CD11b^{LOW} DCs is shown (left). Flow plot is gated on CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{HI}MHCII^{HI} cells. IL-12 production from CD11b^{HI} (middle, top) and CD11b^{LOW} DC (middle, bottom) populations are shown and quantified (right).

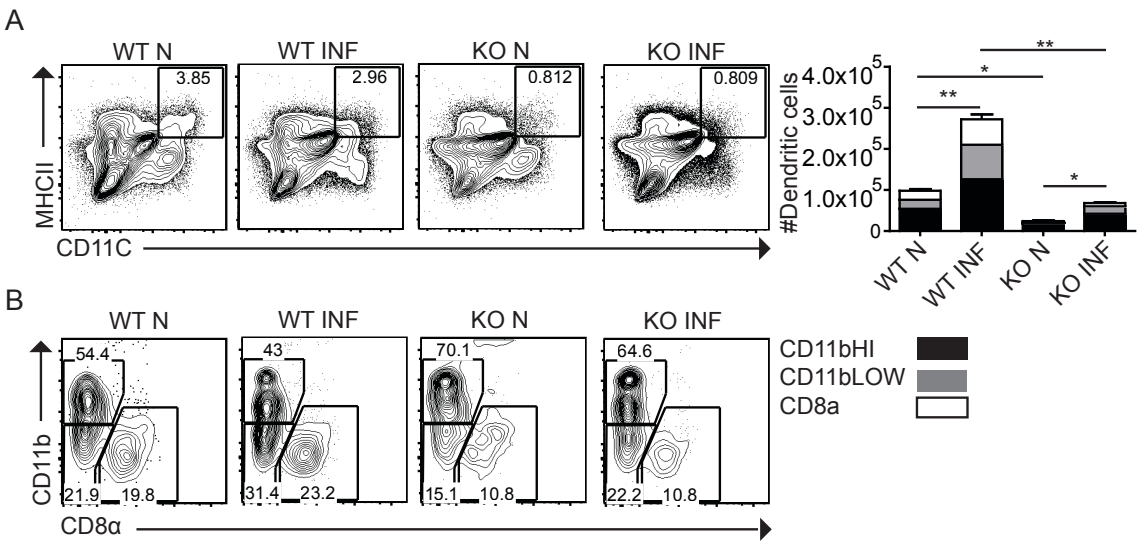

Figure 2.4. Expansion of splenic DC populations in WT and Flt3L KO mice. Splenic DC populations analyzed 8 days post-infection with 10^3 tachyzoites. A. Percentages and numbers of splenic DCs. Flow plots are gated on $CD3^{-ve}CD19^{-ve}NK1.1^{-ve}$ cells. Numbers of splenic DCs are quantified. B. The composition of DC populations in naïve and infected WT and Flt3L KO mice is shown. Flow plots are gated on $CD3^{-ve}CD19^{-ve}NK1.1^{-ve}CD11c^{HI}MHCII^{HI}$ cells.

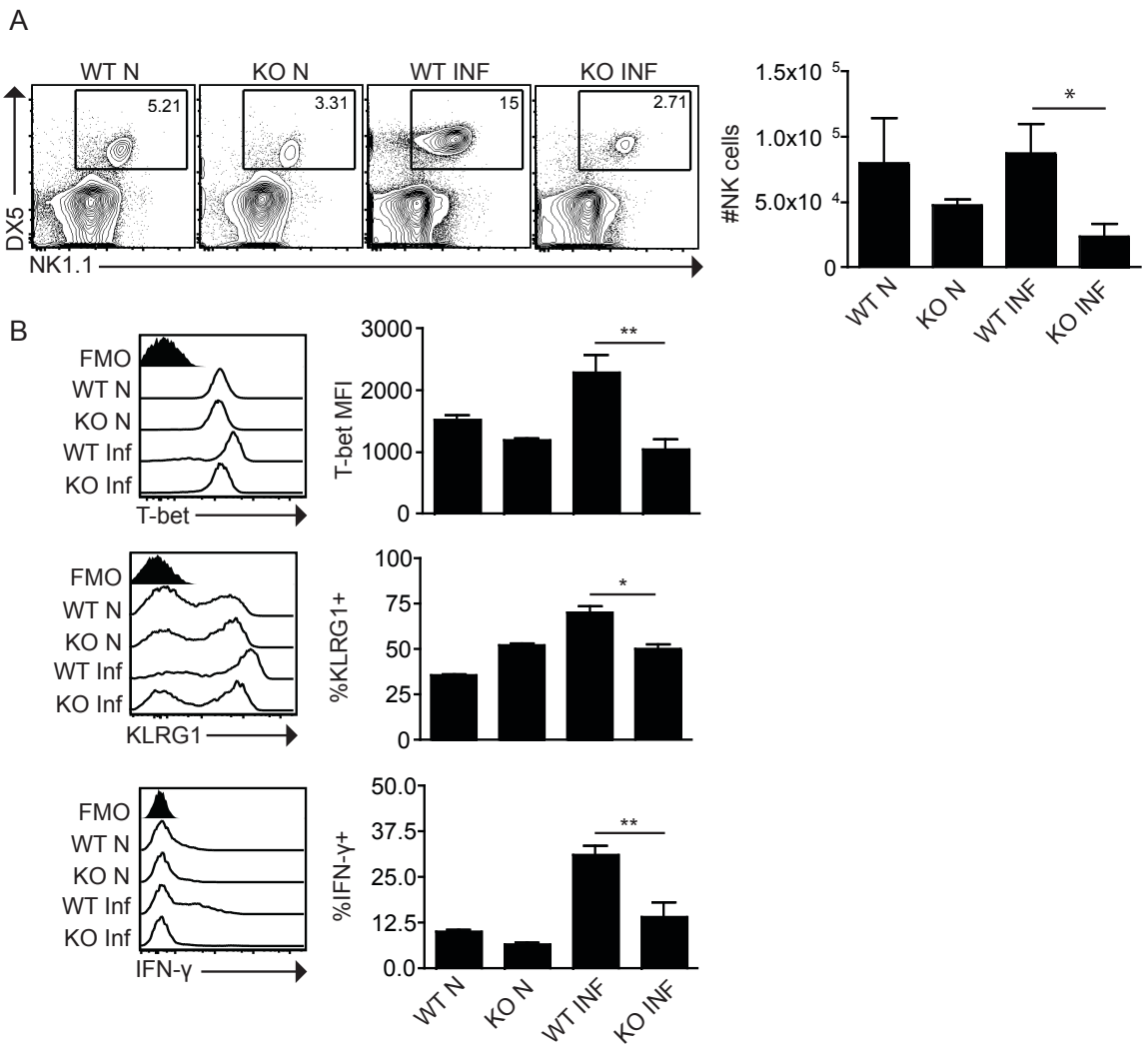

Figure 2.5. Natural killer cell responses in WT and Flt3L KO mice. Natural killer cell responses were examined 5 days post-infection with 10^3 tachyzoites. A. Numbers and percentages of NK cells in the PECS are shown. Similar trends were apparent in the spleen. Flow plots are gated on $CD3^{-ve}$ cells. B. Expression of T-bet, KLRG1 and IFN-γ on splenic NK cells is shown.

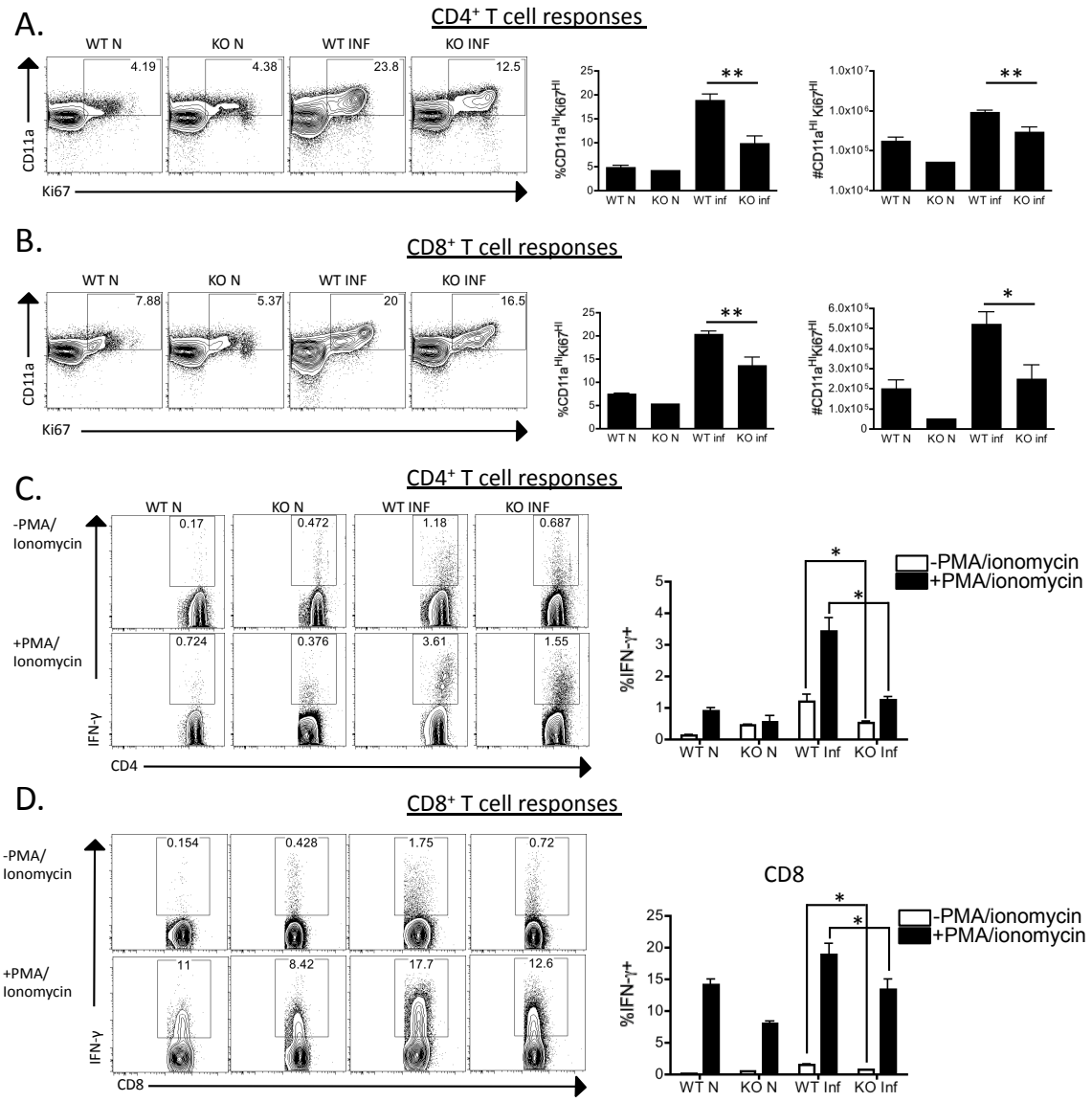

Figure 2.6. CD4$^+$ and CD8$^+$ T cell responses to *T. gondii* in WT and Flt3L KO mice. WT and Flt3L KO mice were infected with 10^3 tachyzoites and CD4$^+$ and CD8$^+$ T cell responses were examined 8 days post-infection. A,B. Splenic CD4$^+$ (A) and CD8$^+$ (B) T cell responses were measured using expression of CD11a and Ki67. Flow plots are gated on CD3$^+$CD4$^+$ (A) or CD3$^+$CD8$^+$ (B) T cells. C,D. Spleenocytes were incubated for 4 hours with Brefeldin A, in the presence or absence of PMA and ionomycin. IFN-γ production was measured from the CD4$^+$ (C) and CD8$^+$ (D) T cells by flow cytometry. Flow plots are gated on CD3$^+$CD4$^+$ (C) or CD3$^+$CD8$^+$ (D) T cells.

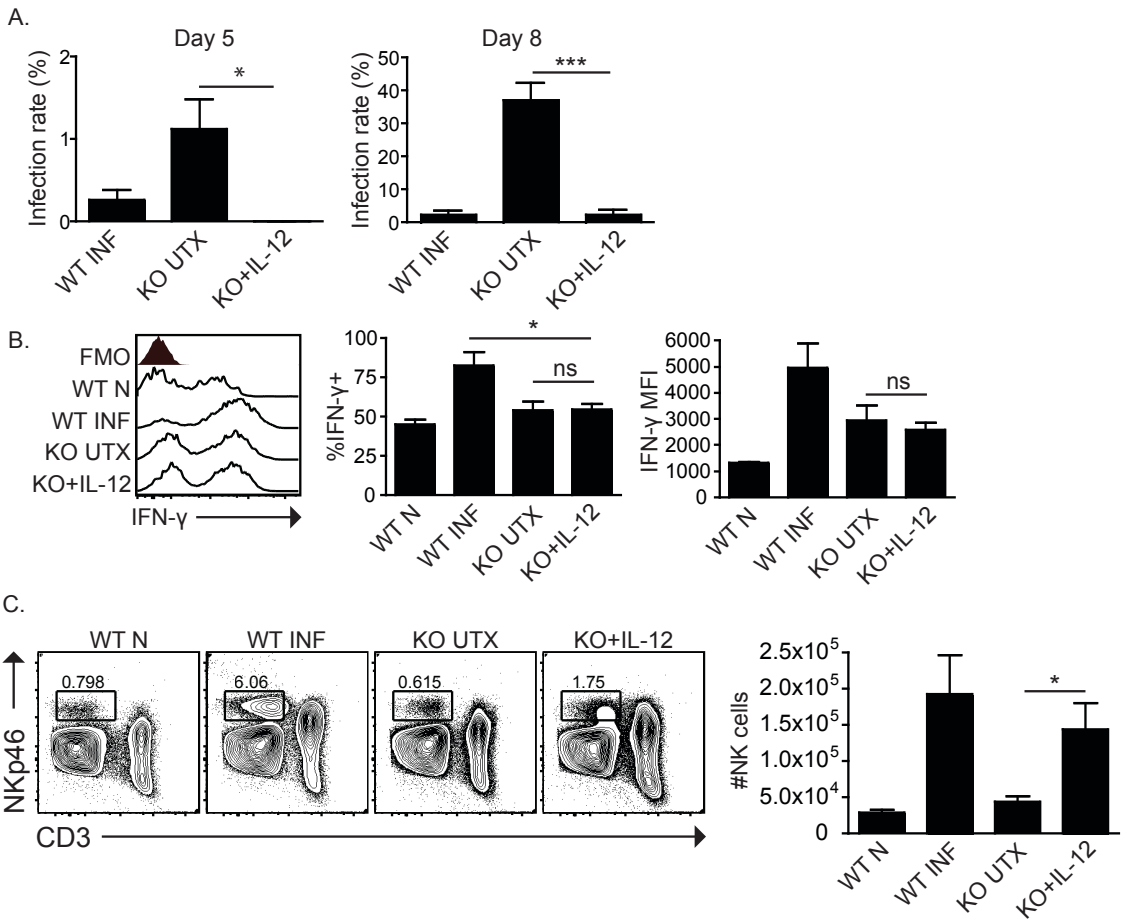

Figure 2.7. IL-12 treatment can decrease parasite burden and partially restore natural killer cell responses in Flt3L KO mice. Flt3L KO mice or WT mice were infected with 10^3 tachyzoites and IL-12 was administered on days 0-3 post-infection for experiments with an endpoint of day 5 post-infection, and days 0-3,5 and 7 for experiments with an endpoint of day 8 post-infection. A. Parasite burden in the peritoneal cavity at days 5 and 8 post infection, as measured by flow cytometry using fluorescently-labeled parasites, or by cytospin analysis. B. IFN-γ production from peritoneal NK cells in WT and Flt3L KO mice, with or without treatment with exogenous IL-12. Flow plots are gated on $CD3^{-ve}NKp46^{+ve}$ cells. Percentages of IFN-γ^{+ve} cells and the MFI of the IFN-γ^{+ve} population are shown. C. Quantification of natural killer cells in the PECS of WT and Flt3L KO mice. Flow plots are gated on live cells, and granulocytes and macrophages have been excluded by forward and side scatter.

CHAPTER 3: PARASITE FATE AND EARLY EVENTS INVOLVED IN THE GENERATION OF $CD4^+$ AND $CD8^+$ T CELL RESPONSES

Abstract

During infection with the intracellular parasite *Toxoplasma gondii*, the presentation of parasite-derived antigens to $CD4^+$ and $CD8^+$ T cells is essential for long-term resistance to this pathogen. Fundamental questions remain regarding the roles of phagocytosis and active invasion in the events that lead to the processing and presentation of parasite antigens. To understand the most proximal events in this process, an attenuated non-replicating strain of *T. gondii* (the *cpsII* strain) was combined with a flow cytometry-based approach to distinguish active invasion from phagocytic uptake. In vivo studies revealed that *T. gondii* preferentially infected dendritic cells, and infected dendritic cells displayed an activated phenotype characterized by enhanced levels of CD86 compared to cells that had phagocytosed the parasite, thus suggesting a role for infected dendritic cells in priming naïve T cells. Indeed, dendritic cells were required for optimal $CD4^+$ and $CD8^+$ T cell responses, and the phagocytosis of heat-killed or invasion-blocked parasites was not sufficient to induce T cell responses. Rather, the selective transfer of *cpsII*-infected dendritic cells to naïve mice potently induced $CD4^+$ and $CD8^+$ T cell responses, and conferred protection against challenge with virulent *T. gondii*. Collectively, these results point toward a critical role for actively infected host cells in initiating *T. gondii*-specific $CD4^+$ and $CD8^+$ T cell responses.

Introduction

As described in Chapter 1 of this thesis, successful control of infection with *Toxoplasma gondii* requires a rapid T_H1 immune response, characterized by the production of the cytokine IL-12, which promotes the ability of parasite-specific $CD4^+$ and $CD8^+$ T cells to produce the cytokine Interferon-γ (IFN-γ) (Gazzinelli et al., 1992a; Gazzinelli et al., 1994; Suzuki et al., 1988). The initiation of $CD8^+$ T cell responses is a complex process which requires that professional antigen presenting cells acquire antigens and present them in the context of Major Histocompatibility Complex (MHC) I, and multiple models have been proposed to explain how this may occur during toxoplasmosis (Dupont et al., 2012; Goldszmid and Sher, 2010). For example, in other systems, foreign antigens are acquired through the pinocytosis of soluble antigens, the phagocytosis of large particulate antigens, or the phagocytosis of host cells containing foreign antigens, and subsequently presented to $CD8^+$ T cells through cross-presentation (Brode and Macary, 2004; Steinman et al., 2003). A role for cross presentation during toxoplasmosis is supported by in vivo imaging studies showing that uninfected dendritic cells interact extensively with parasite-specific $CD8^+$ T cells (Chtanova et al., 2009; Goldszmid and Sher, 2010; John et al., 2009). Alternatively, since *T. gondii* is an intracellular parasite, actively infected dendritic cells may acquire parasite-derived antigens from their intracellular environment independently of phagocytosis and directly prime naïve $CD8^+$ T cells. Indeed, the ability of cells actively infected by *T. gondii* to prime or present antigen to $CD8^+$ T cells has been observed in vitro (Dzierszinski et al., 2007; Goldszmid et al., 2009; Gubbels et al., 2005) and the critical role of perforin in immunity to *T. gondii* implicates the cytolysis of infected host cells as a mechanism of defense, thus arguing that infected cells can present antigen to effector $CD8^+$ T cells in vivo (Denkers et al., 1997). However, several caveats must be acknowledged in interpreting these studies. Firstly, the ability of infected cells to present antigens to reporter cells lines or activated effector $CD8^+$ T cells does not necessarily indicate that infected cells can prime naïve $CD8^+$ T cells, and events that occur in vitro may not represent the in vivo situation. Additionally, it can be difficult to distinguish actively infected host cells from cells that have phagocytosed the parasite by flow cytometry, thus confounding experimental interpretation. Furthermore, like many intracellular pathogens, *T. gondii*

has been reported to inhibit the expression or upregulation of molecules involved in antigen presentation such as MHCI, CD40, CD80, and CD86 in infected cells, suggesting that the ability of the ability of infected cells to prime naïve $CD8^+$ T cells may be compromised (Luder et al., 1998; Luder et al., 2001; McKee et al., 2004).

Antigens presented to $CD4^+$ T cells in the context of MHCII may also be derived from the extracellular or intracellular environment of the host cell. Endocytosed antigens can be presented in the context of MHCII, and this pathway is considered to be the primary mechanism by which antigens are acquired for presentation to $CD4^+$ T cells (Neefjes et al., 2011). However, intracellular antigens can also be presented in the context of MHCII, as cytosolic peptides are presented in the context of MHCII by B cells and macrophages (Dongre et al., 2001). Similarly, in vitro studies have demonstrated that viral or model antigens expressed intracellularly can be presented to $CD4^+$ T cells independently of phagocytosis (Aichinger et al., 1997; Bonifaz et al., 1999; Jaraquemada et al., 1990; Lich et al., 2000; Malnati et al., 1992; Nimmerjahn et al., 2003; Nuchtern et al., 1990; Paludan et al., 2005; Weiss and Bogen, 1991). Despite these findings, the role of infected cells in presenting antigen to $CD4^+$ T cells in vivo during any infection remains unclear (Iwasaki and Medzhitov, 2010). In the case of *T. gondii*, downregulated expression of MHCII and other molecules involved in antigen presentation has been observed on infected cells, and cells infected with *T. gondii* exhibit decreased ability to present antigen in vitro (Luder et al., 1998; Luder et al., 2001; McKee et al., 2004). Furthermore, in vitro studies have observed that antigens from heat-killed or invasion-inhibited parasites incubated with dendritic cells can be presented in the context of MHCII, consistent with a role for phagocytosis-dependent antigen presentation to $CD4^+$ T cells (Goldszmid et al., 2009).

There are several difficulties involved with addressing the relative contributions of phagocytosis versus active invasion to antigen presentation in vivo during many infections. For example, interfering with these pathways can result in changes in pathogen burden and inflammation that confound experimental interpretation, and the parasite-mediated lysis of host cells and re-infection may obscure the analysis of the earliest cell populations that interact with the pathogen. In addition, there are limited tools to distinguish host cells that have phagocytosed

pathogens from those that have been productively infected. In the present study, these issues are addressed using a non-replicating uracil auxotrophic vaccine strain of *T. gondii* (the *cpsII* strain) (Fox and Bzik, 2002; Wilson et al., 2010; Wilson et al., 2008) and a novel assay that tracks the fate of parasites and distinguishes active invasion from phagocytosis in vivo. Using these approaches, *cpsII* parasites were found to infect large numbers of macrophages and to preferentially infect dendritic cells, and dendritic cells were found to be necessary for optimal *cpsII*-induced $CD4^+$ and $CD8^+$ T cell responses. Infected dendritic cells displayed an activated phenotype, characterized by high levels of CD86 and MHCI expression, which was unique from the phenotype of dendritic cells that had phagocytosed *T. gondii*. Furthermore, the administration of heat-killed or invasion blocked parasites did not induce $CD4^+$ or $CD8^+$ T cell responses, thus demonstrating that phagocytosis of parasites is insufficient to activate naïve T cells. Lastly, the selective transfer of infected dendritic cells to naïve mice resulted in robust $CD4^+$ and $CD8^+$ T cell responses and protection from challenge with a virulent strain of *T. gondii*. These findings point toward a critical role for infected cells in initiating the adaptive immune response to *T. gondii*.

Materials and Methods

Ethics Statement All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Animal Welfare Assurance Reference Number #A3079-01) and were in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

Mice Flt3L KO mice were obtained from Taconic Farms (Germantown, NY). Batf3 KO mice and CD11c-DTR mice were obtained from Jackson Laboratories. C57BL/6 mice were obtained from Jackson Laboratories or Taconic Farms. All mice were kept in specific-pathogen-free conditions at the School of Veterinary Medicine at the University of Pennsylvania. For experiments in which dendritic cells were depleted, CD11c-DTR or WT control mice were administered 100 ng of Diphtheria Toxin (Sigma-Aldrich) diluted in 100 µL of PBS (Invitrogen) intraperitoneally ~12 hours prior to vaccination. Depletion efficiency was typically 90%.

Infections All experiments were performed using *cpsII* parasites, *cpsII*-OVA parasites (Jordan et al., 2009), *cpsII*-OVA-mCherry parasites, or RH-OVA-Tomato parasites. RH-OVA-Tomato parasites (Whitmarsh et al., 2011) and *cpsII*-OVA parasites (Jordan et al., 2009; Pepper et al., 2004) have been previously described, and *cpsII*-OVA parasites were derived from the RHΔ*cpsII* clone, which was provided as a generous gift by Dr. David Bzik (Fox and Bzik, 2002). *CpsII*-OVA-mCherry parasites were derived from the *cpsII*-OVA clone using the previously described methods (Koshy et al., 2012; Koshy et al., 2010), with the exception that parasites were selected using zeomycin as previously described (Messina et al., 1995). Parasites were cultured and maintained by serial passage on human foreskin fibroblast cells in the presence of parasite culture media [71.7% DMEM (Corning), 17.9% Medium 199 (Invitrogen), 9.9% Fetal Bovine Serum (FBS)(Invitrogen), 0.45% Penicillin and Streptomycin (Invitrogen)(final concentration of 0.05 units/ml Penicillin and 50 µg/ml Streptomycin), 0.04% Gentamycin (Invitrogen)(final concentration of 0.02 mg/ml Gentamycin)], which was supplemented with uracil (Sigma-Aldrich)(final concentration of 0.2 mM uracil) in the case of *cpsII*, *cpsII*-OVA and *cpsII*-OVA-mCherry parasites. For infections, parasites were harvested and serially passaged through 18, 20 and 26 gauge needles (BD) before filtration with a 5 µM filter (Sartorius Stedim). Parasites were washed extensively with PBS and mice were injected intraperitoneally with 10^5 or 10^6 parasites suspended in PBS. In vitro experiments were performed at an MOI of 0.5 or 1. For experiments in which CellTrace™ Violet (Invitrogen) was utilized to track the fate of parasites, CellTrace™ Violet was diluted in 200 µL of DMSO to obtain a 0.5 mM stock solution. Parasites were washed once with PBS before incubation in 0.5 µM CellTrace™ Violet diluted in PBS for 10-25 minutes at 37^O C. This reaction was quenched by the addition of ~40 volumes of complete media [88.5% RPMI 1640 (Corning), 8.8% FBS (Invitrogen), 0.9% Sodium Pyruvate (Gibco), 0.9% Penicillin and Streptomycin (Invitrogen)(final concentration of 0.1 units/ml Penicillin and 100 µg/ml Streptomycin), 0.9% MEM Non-essential Amino Acids Solution (Gibco) and 0.18% beta-2-mercaptoethanol (Gibco)] and parasites were washed extensively. In experiments in which 4-p-bromophenacyl bromide (4-p-bpb) was utilized to inhibit parasite invasion, 4-p-bpb (Sigma-Aldrich) was prepared fresh for each experiment and dissolved in DMSO (Sigma-Aldrich) to make

a 0.1 M stock solution. Parasites were incubated in a 100 μM solution of 4-p-bpb in Fetal Bovine Serum at a concentration of 10^7 parasites/ml for 10 minutes, and the reaction was quenched by the addition of ~40 volumes of complete media, followed by extensive washing (Goldszmid et al., 2009). To heat-kill parasites, parasites were incubated at 60^{O}C for 1 hour in PBS (Haque et al., 1999). Death was confirmed using Trypan Blue staining (Corning).

Cell culture and tissue harvesting Peritoneal exudate cells were obtained by peritoneal lavage with 5 ml of PBS. Splenocytes and lymphocytes were obtained by grinding spleens and lymph nodes over a 40 μM filter (Biologix) and washing them in complete media. Red blood cells were then lysed by incubating for 5 minutes at room temperature in 5 ml of lysis buffer [0.864% ammonium chloride (Sigma-Aldrich) diluted in sterile de-ionized H_2O)], followed by washing with complete media. Bone marrow-derived macrophages were obtained using previously described methods (Robben et al., 2004; Whitmarsh et al., 2011). Immortalized macrophages from C57BL/6 mice were obtained by transforming bone marrow derived macrophages with the J2 Virus and were cultured in macrophage media (Blasi et al., 1989).

Flow Cytometry and imaging Tetramer-specific $CD4^+$ T cells were measured using the protocol previously described (Pepper et al., 2011). MHCII Tetramer was obtained as generous gifts from Drs. Marc Jenkins and Marion Pepper, and subsequently from the NIH Tetramer Core Facility, and was used at a final concentration of 10 nM. APC-MHCI-SIINFEKL Tetramer was obtained from Beckman-Coulter. Cells were washed with FACS Buffer [1x PBS, 0.2% bovine serum antigen (Sigma), 1 mM EDTA (Invitrogen)], stained with LIVE/DEAD® Fixable Aqua Dead Cell marker (Invitrogen) and incubated in Fc block [99.5% FACS Buffer, 0.5% normal rat serum (Invitrogen), 1 μg/ml 2.4G2 (BD)] prior to staining. The following antibodies were used for staining: Ki67 Alexa Fluor®488 (BD, B56), CD3 APC-eFluor®780 (eBioscience, 17A2), CD8 eFluor®450 (eBioscience, 53-6.7), CD11a PerCP-Cy5.5 (Biolegend, H155-78), MHCII PE (eBioscience, M5/114.15.2), NK1.1 PE (BD, PK136), CD19 PE (eBioscience, 1D3), Foxp3 eFluor®450 (eBioscience, FJK-16a), CD4 Pe-Cy7 (eBioscience, GK1.5), CD3 FITC (BD, 145-2C11), NK1.1 FITC (eBioscience, PK136), CD19 FITC (eBioscience, 1D3), Gr-1 PerCP-Cy5.5 (eBioscience, RB6-8C5), CD11c PE-Cy7 (eBioscience, N418), CD11b APC-eFluor®780

(eBioscience, M1/70), MHCII AF700 (Biolegend, M5/114.15.2), MHCI APC (AlexaFluor®647 AF6-88.5), CD86 APC (eBioscience, GL1), CD40 APC (eBioscience 1C10), CD8 eFluor®650 NC (eBioscience, 53-6.7), CD45.2 APC-eFluor®780 (eBioscience, 104), polyclonal rabbit anti-*T. gondii* [a generous gift from Fausto G. Araujo (Palo Alto Medical Foundation, Palo Alto, CA)], and polyclonal Goat anti-Rabbit Alexa Fluor® 680 (Jackson). Intracellular staining was performed using the Foxp3/ Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer's instructions. Samples were run on a FACSCanto (BD) or LSR Fortessa (BD) and analyzed using FlowJo Software (TreeStar). Images were obtained using the ImageStream and analysis was performed using IDEAS software (Amnis).

Sorting Splenic dendritic cells were obtained from mice injected subcutaneously with Flt3L-secreting b16 tumor cells (Curran and Allison, 2009; Maraskovsky et al., 1997) and magnetically enriched using CD11c microbeads (Miltenyi Biotech) and LD MACS® separation columns (Miltenyi Biotech), following the manufacturer's instructions. Following enrichment, dendritic cells were cultured overnight with parasites at 37^{O} C and collected the following day. Dendritic cells were then stained for MHCII, CD11c, CD45, and free parasites, and sorted for $mCherry^{+ve}Violet^{+ve}$ or $mCherry^{-ve}Violet^{-ve}$ populations that were $CD45^{+}MHCII^{HI}CD11c^{HI}$, and negative for free parasites using the FACSAria (BD).

Electron microscopy Bone marrow derived macrophages from C57BL/6 mice were activated with IFN-γ and LPS for 18-24 hours or left untreated in macrophage media lacking uracil [DMEM (Gibco) supplemented with 4mM L-glutamine (Sigma) and 10% dialyzed fetal bovine serum (Hyclone)]. Where indicated, cells were infected with freshly egressed parasites, washed three times with PBS then fixed at 2 hours or 24 hours post infection. For ultrastructural analysis, cells were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM phosphate buffer, pH 7.2 for 1 hour at room temperature, processed and examined as described previously (Fentress et al., 2010).

Immunofluorescence assays Immunofluorescence assays were performed in RAW 264.7 macrophages. Cells were activated as indicated with IFN-γ and LPS for 18-24 hours in macrophage media lacking uracil. Macrophages were infected with freshly egressed parasites at

an MOI of 1, washed at 30 minutes post infection three times with PBS, and incubated in uracil-free media for the indicated time. Cells for immunofluorescence were fixed in 4% formaldehyde, permeabilized with 0.05% saponin, and stained using primary antibodies as described. Parasite vacuoles were localized using mouse monoclonal Tg17-43 against GRA1 or rabbit polyclonal sera against GRA7. Host LAMP-1 was localized with rat monoclonal antibody 1D4B and Irgb6 was localized using rabbit polyclonal sera raised against recombinant protein (Henry et al., 2009). All secondary antibodies used in immunofluorescence were highly-cross adsorbed Alexa Fluor conjugated antibodies (Invitrogen). Samples were visualized using a Zeiss Axioskop 2 MOT Plus microscope equipped for epifluorescence and using a 63X Plan Apochromat lens, N.A. 1.40 (Carl Zeiss, Inc., Thornwood, NY). Images were acquired with an AxioCam MRm camera (Carl Zeiss, Inc.) using Axiovision v4.6, and processed using similar linear adjustments for all samples in Photoshop CS4 v9.

Statistical Analysis Statistical analysis was performed using PRISM software (Graphpad Software). Significance was calculated using an unpaired two-tailed student's t-test except when otherwise noted.

Results

Development of a system to distinguish phagocytosis of parasites from active invasion

To distinguish between parasites that are phagocytosed by host cells and those that actively infect host cells, differences in sensitivity to pH between the fluorescent markers mCherry and CellTrace™ Violet were exploited. When mCherry-expressing parasites were labeled intracellularly with CellTrace™ Violet and incubated overnight in buffer solutions of varying pH, mCherry fluorescence was retained (Figure 3.1a). In contrast, violet fluorescence intensity was maintained at pH 7.0 but was decreased at low pH (Figure 3.1a). The ability of this system to distinguish active invasion from phagocytosis was demonstrated in vitro by incubating Violet-labeled, mCherry-expressing *cpsII* parasites with macrophages and examining fluorescence by flow cytometry 1 hour and 18 hours post infection. At one hour after incubation with parasites, two distinct macrophage populations were present: One displayed mCherry and Violet fluorescence,

while the other was negative for both markers (Figure 3.1b). However, by 18 hours, two distinct mCherry^{+ve} populations were apparent. One population displayed no loss of mCherry or Violet fluorescence (mCherry^{+ve}Violet^{+ve}), while the other population had decreased mCherry fluorescence associated with a complete loss of violet fluorescence (mCherry^{+ve}Violet^{-ve}). Utilizing ImageStream® flow cytometry to generate images of individual cells from each of these populations revealed that the mCherry^{+ve}Violet^{+ve} cells contained intact parasites, while the mCherry^{+ve}Violet^{-ve} cells contained dimmer and more diffuse mCherry fluorescence (Figure 3.1c, Figure 3.S1-3). Furthermore, pre-treatment of parasites with the irreversible inhibitor of invasion 4-p-bromophenacyl bromide (4-p-bpb) (thus making parasites targets for phagocytosis) (Goldszmid et al., 2009; Morgado et al., 2011; Ravindran et al., 2009; Saffer et al., 1989), resulted in the complete loss of the mCherry^{+ve}Violet^{+ve} population at 18 hours post-infection (Figure 3.1b,c, Figure 3.S1-3). Collectively, these results are consistent with a model in which phagocytosed parasites are degraded, and the acidic environment of the phagosome leads to a loss of Violet fluorescence, while mCherry fluorescence is retained. In contrast, when the parasite actively invades host cells and persists in the less acidic environment of the parasitophorous vacuole (PV), both Violet and mCherry fluorescence are retained.

The ability to distinguish active invasion from phagocytosis was then utilized to determine the fate of *cpsII* parasites in vivo. When C57Bl/6 mice were vaccinated intraperitoneally with Violet-labeled, mCherry-expressing parasites, mCherry^{+ve}Violet^{+ve} and mCherry^{+ve}Violet^{-ve} populations were apparent in the Peritoneal Exudate Cells (PECS) 18 hours post-vaccination, and the presence of the mCherry^{+ve}Violet^{+ve} population was abrogated by pre-treating the parasites with 4-p-bpb (Figure 3.1d). Furthermore, when Violet^{+ve} cells were sorted and cytospins examined, they were found to contain intact parasites (Figure 3.1e). ImageStream analysis also revealed that the mCherry^{+ve}Violet^{+ve} population contained intact parasites whereas the mCherry^{+ve}Violet^{-ve} population displayed diffuse mCherry fluorescence (Figure 3.1f, Figure 3.S4-6). Collectively, these studies demonstrate that the use of fluorescent markers with differing pH sensitivities can be used to distinguish cells that have phagocytosed *T. gondii* from those that have been actively infected.

CpsII parasites persist in actively infected host cells

Because many *cps*II parasites were found to actively invade host cells, the fate of these parasites was then analyzed over time using electron microscopy and immunofluorescence assays to determine the integrity of the PV and the sub-cellular localization of the parasite. Immunofluorescence microscopy revealed that parasites can persist in infected cells up to forty-eight hours following infection, in both macrophages activated with IFN-γ and LPS, as well as untreated macrophages (Figure 3.2a,b). Because IFN-γ can induce the recruitment of immune enzymes such as the Immunity Related Guanosine Triphosphatases (IRGs) to the PV, and these enzymes have been implicated in the rupture of the PV which leads to the xenophagic elimination of the parasite, the colocalization of the parasite with Irgb6 (a member of the IRG family) and LAMP-1 (which is expressed on lysosomes) was examined in macrophages pre-treated with IFN-γ and LPS. *CpsII* parasites were not found to colocalize with either Irgb6 or LAMP-1, arguing against a role for the IRG-mediated and xenophagic elimination of *cpsII* parasites, respectively (Figure 3.2a,b). Electron microscopy was also utilized to examine the integrity of the PV, since IFN-γ can induce the blebbing and rupture of the PV during infection with replicating strains of *T. gondii* (Ling et al., 2006; Martens et al., 2005). Using electron microscopy, no IFN-γ induced blebbing or rupture of the PV was apparent in *cpsII*-infected (Figure 3.2c). Additionally, some *cpsII* parasites showed atypical morphology, indicative of non-productive cell division (Figure 3.2d). To measure the persistence of *cpsII* parasites in vivo, bioassays were performed in which tissues from vaccinated mice were cultured in the presence of exogenous uracil and examined by microscopy for the presence of *cpsII* parasites. Using this method, *cpsII* parasites were found to persist in the peritoneal cavity up to five days post-vaccination (data not shown). These results confirm that *cpsII* parasites cannot replicate within host cells, and suggest that *cpsII* parasites can persist for long periods of time, evading IFN-γ-mediated destruction.

Identification and phenotypic analysis of cells that are infected by or phagocytose *cpsII* parasites

To better understand the fate of *cpsII* parasites in vivo, mice were challenged intraperitoneally with Violet-labeled, mCherry-expressing *cpsII* parasites, and flow cytometry was performed on the PECS 18 hours later to characterize the cell populations that had phagocytosed *T. gondii* or were actively infected. The largest population of $mCherry^{+ve}Violet^{+ve}$ cells to be infected was $CD11b^{HI}$ macrophages, which comprised 44±16.7% of infected cells. Dendritic cells (which have been previously implicated in the induction of T cell responses to *cpsII* (Gigley et al., 2009)) comprised 8.3±2.8% of infected cells (Figure 3.3a). Of the infected dendritic cells the vast majority (97.8±2%) belonged to the $Gr\text{-}1^{-ve}CD11b^{HI}$ subset (data not shown). Although *T. gondii* is capable of infecting any nucleated cell, dendritic cells were overrepresented in the infected population, suggesting that this population is preferentially infected by the parasite (Figure 3.3b). Analysis of the population that had phagocytosed *T. gondii* revealed 46.0±20.6% of these cells were $CD11b^{HI}$ macrophages, whereas dendritic cells represented 6.2±3.2% of this population (Figure 3.3a). Parasites were not detected in lymph nodes or spleens by flow cytometry, and parasites could not be cultured from these tissues at days 3,5 or 10 post-vaccination (data not shown).

The phenotype of infected cells and cells that phagocytosed the parasite was compared by analyzing expression levels of MHCI and MHCII, as well as the costimulatory molecules CD86 and CD40. Although vaccination with *cpsII* resulted in an overall increase in expression of MHCI on $CD11b^{HI}$ macrophages, macrophages that had phagocytosed the parasite and those that were infected displayed similar MHCI levels to the total population of macrophages in the PECS of vaccinated mice. In contrast, dendritic cells that had phagocytosed *cpsII* and those that were infected by the parasite displayed higher levels of MHCI relative to the total dendritic cell population in the peritoneal cavity (Figure 3.4a). Vaccination with *cpsII* induced no significant changes in MHCII expression of $CD11b^{HI}$ macrophages or dendritic cells on any population that was examined (Figure 3.4b). Expression of CD86 was markedly higher on macrophages and dendritic cell populations that were infected by the parasite, but not the populations that had

phagocytosed the parasite (Figure 3.4c). While vaccination induced increased CD40 expression on the total dendritic cell population, infected cells displayed similar expression levels to the total population, and those that phagocytosed the parasite exhibited the highest levels of expression (Figure 3.4d). Collectively, these results demonstrate that infected dendritic cells display an activated phenotype, characterized by the upregulation of MHCI and CD86, and expression of CD40 and MHCII, which is unique from the phenotype of cells that have phagocytosed *T. gondii*.

Dendritic cells are critical for optimal *cpsII*-induced $CD4^+$ and $CD8^+$ T cell responses

Given the activated phenotypes of dendritic cells infected with *cpsII* and those that had phagocytosed the parasite, studies were performed to determine the role of dendritic cells in the development of $CD4^+$ and $CD8^+$ T cell responses to this strain. Mice that express the diphtheria toxin receptor under the control of the CD11c promoter (CD11c-DTR mice) were used to test the requirement for dendritic cells to prime T cells (Jung et al., 2002). In these experiments, CD11c-DTR mice were treated with diphtheria toxin, which resulted in a 70-90% reduction in dendritic cells (Figure 3.5a). One day following the administration of diphtheria toxin, mice were challenged with a strain of *cpsII* engineered to express Ovalbumin (*cpsII*-OVA) (Jordan et al., 2009). At eight days following vaccination, $CD4^+$ and $CD8^+$ T cell responses were measured using MHCII tetramers which bind $CD4^+$ T cells specific for the endogenous *T. gondii* epitope CD4Ag28m combined with magnetic enrichment for the $tetramer^{+ve}$ population (Grover et al., 2012; Pepper et al., 2011), and MHCI tetramers for OVA-specific CD8+ T cells. Additionally, the surface molecule CD11a, which is upregulated on antigen-experienced $CD4^+$ and $CD8^+$ T cells (McDermott and Varga, 2011; Rai et al., 2009), and the intracellular molecule Ki67, which is indicative of cellular proliferation (Scholzen and Gerdes, 2000) were used to measure the total $CD4^+$ and $CD8^+$ T cell responses to *T. gondii*. Indeed, vaccination with *cpsII* induced a two-fold increase in the frequency of $CD11a^{HI}Ki67^{HI}$ cells and an increase in the number of $CD11a^{HI}$ $CD4^+$ T cells specific for the CD4Ag28m epitope, but depletion of dendritic cells inhibited these responses (Figure 3.5b). Similarly, *cpsII* vaccination induced an increase in $CD11a^{HI}$ $Ki67^{HI}$ and OVA-specific $CD8^+$ T cells, however these responses were decreased in mice depleted of dendritic cells (Figure

3.5c). Furthermore, when Flt3L-deficient mice (which have global defects in numbers of dendritic cells (McKenna et al., 2000)) or Batf3-deficient mice (which have a defect in numbers of $CD8a^{+}$ dendritic cells (Hildner et al., 2008)) were challenged with *cpsII*-OVA, both mice displayed marked defects in tetramer-specific and total $CD4^{+}$ and $CD8^{+}$ T cell responses (Figures 3.S7, 3.S8). Collectively, these results establish a role for dendritic cells in the generation of $CD4^{+}$ and $CD8^{+}$ T cell responses following *cpsII* vaccination.

Infected dendritic cells are sufficient to generate $CD4^{+}$ and $CD8^{+}$ T cell responses

To assess the contribution of phagocytosis to the generation of $CD4^{+}$ and $CD8^{+}$ T cell responses, mice were challenged with live *cpsII*-OVA parasites, heat-killed *cpsII*-OVA parasites, or parasites pre-treated with the irreversible inhibitor of invasion 4-p-bpb. As expected, vaccination with live parasites induced the $tetramer^{+ve}$ and $tetramer^{-ve}$ $CD4^{+}$ T cell responses, however these responses were abrogated when parasites were killed or invasion was inhibited (Figure 3.6a). Similarly, $CD11a^{HI}Ki67^{HI}$ and OVA-specific $CD8^{+}$ T cells were detected when mice were administered live, but not heat-killed or invasion-inhibited parasites (Figure 3.6b). Indeed, even when the dose of heat-killed parasites was increased to 10^{7} parasites (100x the typical dose of live parasites), no $CD4^{+}$ or $CD8^{+}$ T cell responses could be detected (data not shown). Additionally, gp91 KO mice, which have a defect in cross-presenting antigens to $CD8^{+}$ T cells (Savina et al., 2006), developed normal $CD8^{+}$ T cell responses following *cpsII*-vaccination (data not shown). Collectively, these data indicate that phagocytosis of parasites is insufficient to induce $CD4^{+}$ and $CD8^{+}$ T cell responses, and point toward a critical role for infected cells in these processes.

To directly test whether actively infected host cells induce $CD4^{+}$ and $CD8^{+}$ T cell responses, splenic dendritic cells were infected with violet-labeled, mCherry-expressing *cpsII* parasites in vitro overnight, and FACS sorting was used to purify the uninfected ($mCherry^{-ve}Violet^{-ve}$) and infected cells ($mCherry^{+ve}Violet^{+ve}$) cells from the same cultures, and each of these fractions was then administered to naïve mice. Consistent with the in vivo data, dendritic cells that phagocytosed *T. gondii* in vitro comprised a small percentage of the total population, and it was

not feasible to isolate this population by sorting. In mice administered uninfected dendritic cells there was no detectable increase in proliferating, antigen-experienced $CD4^+$ or $CD8^+$ T cells (Figure 3.7a,b). In contrast, mice administered *cpsII*-infected dendritic cells developed $CD4^+$ and $CD8^+$ T cell responses as determined by tetramer-binding as well as expression of Ki67 and CD11a. Furthermore, when recipients of infected or uninfected dendritic cells were challenged 3 weeks later with a highly virulent strain of *T. gondii* only those mice administered *cpsII*-infected dendritic cells displayed a ~99% reduction in parasite burden (Figure 3.7c). Collectively, these results demonstrate a critical role for infected cells in the induction of protective $CD4^+$ and $CD8^+$ T cell responses.

Discussion

There are many fundamental questions about the mechanisms of antigen presentation that lead to the activation of $CD4^+$ and $CD8^+$ T cells during toxoplasmosis and multiple studies have addressed the ability of actively infected cells to present antigen (Dzierszinski et al., 2007; Goldszmid et al., 2009; Gubbels et al., 2005; Subauste and Wessendarp, 2000). The present work highlights that following challenge in vitro or in vivo with live parasites there are high rates of phagocytosis and conventional methods using parasites that express a single fluorescent reporter protein are not sufficient to determine how an individual cell has interacted with *T. gondii*. Rather, the ability to combine parasites that express a pH insensitive reporter such as mCherry protein with a pH sensitive dye and analysis by high throughput imaging and flow cytometry provide a unique opportunity to examine parasite fate and host cell phenotype. This combination of approaches should be broadly applicable to determining the fate of other intracellular fungal, bacterial and parasitic pathogens (da Silva et al., 2012; Dunn and Valdivia, 2010; Overstreet et al., 2008; Rohde et al., 2007; Romano et al., 2012; Seider et al., 2010; Shin and Roy, 2008). Regardless, the ability to distinguish these fates revealed that macrophages and dendritic cells infected by *T. gondii* have unique activation phenotypes when compared to those that have phagocytosed the parasite. Previous reports have indicated that infection with *T. gondii* inhibits the maturation of professional antigen presenting cells (Goldszmid and Sher, 2010; Lang et al.,

2006; Luder et al., 1998; McKee et al., 2004) but the data presented here are more consistent with the idea that infection induces DC maturation (Baird et al., 2013; Morgado et al., 2011; Subauste et al., 1998; Subauste and Wessendarp, 2000; Walseng et al., 2010).

In current paradigms, the direct phagocytosis or endocytosis of soluble and particulate non-infectious antigens is the major pathway that allows antigens to be presented in the context of MHCII to $CD4^+$ T cells (Neefjes et al., 2011). Similarly, phagocytosed antigens are thought to be presented to $CD8^+$ T cells through the process of cross-presentation (Brode and Macary, 2004). However, the studies using invasion-blocked or heat-killed parasites, and the studies in which infected or uninfected cells were transferred to mice demonstrate that active invasion, rather than phagocytosis is critical to induce T cell responses following *cpsII* vaccination. These data are therefore consistent with models in which infected cells either directly prime $CD4^+$ T and $CD8^+$ T cells, or are taken up by efferocytosis (i.e. the phagocytosis of apoptotic cells), leading to antigen presentation. Both of these models imply a mechanism by which infected cells can sense the parasite, either to upregulate the signals that promote antigen presentation or to induce apoptosis, and the finding that infected cells display an activated phenotype is consistent with the idea that infected cells directly sense the parasite. Indeed, Toll Like Receptor-dependent and independent pathways have been implicated in sensing *T. gondii*, but questions remain regarding which specific host-parasite interactions (e.g. phagocytosis, active invasion, endocytosis of soluble antigens) activate specific sensing mechanisms (Egan et al., 2009b; Morgado et al., 2011; Pifer and Yarovinsky, 2011; Witola et al., 2011). It is also possible that the activated status of infected cells results from the activity of effector proteins secreted by the parasite, which may act independently of molecules traditionally thought of as host pattern recognition receptors (Hunter and Sibley, 2012).

Since *T. gondii* resides in a specialized non-fusogenic vacuole, it is unclear how parasite antigens may escape the PV for processing and presentation. One possibility is that parasite antigens are acquired for presentation from the intracellular environment through the xenophagic elimination of *cpsII* parasites. In support of this possibility, autophagic machinery has been implicated in the elimination of *T. gondii* (Ling et al., 2006; Zhao et al., 2009b; Zhao et al., 2008),

and antigen acquired through autophagy can be subsequently presented (Nimmerjahn et al., 2003; Paludan et al., 2005; Romao et al., 2013). However, the lack of recruitment of Irgb6 and Atg5 to the PVs containing *cpsII* parasites argues against this idea. Antigens may also escape the PV through the fusion of the PV with the endoplasmic reticulum (Goldszmid et al., 2009), may be secreted into the cytoplasm during the invasion process (Koshy et al., 2010), or may leak out of the PV (Gubbels et al., 2005). More recent work has shown that *T. gondii* can secrete antigens into host cells without subsequently infecting these cells (Koshy et al., 2012). This population of injected-but-uninfected cells may also contribute to the host immune response, and the ability to track these abortive invasion events in vivo (Koshy et al., 2010), as well as the ability to divorce injection from infection through modulation of the parasite, may provide further insight into the pathways involved in antigen processing during *cpsII* vaccination. Regardless, the finding that phagocytosis is insufficient to induce antigen presentation in this system highlights the importance of alternative approaches to deliver antigens for vaccine design and immunotherapies, such as those that target antigens to the host cell cytosol (Moon et al., 2012).

Given the lack of overt inflammation observed during infection with *cpsII* parasites, the absence of parasite-driven cytolysis of host cells, and limited antigen load, it was somewhat surprising that relatively low numbers of parasites are able to generate strong protective $CD4^+$ and $CD8^+$ T cell responses, comparable to those seen during live infection (Jordan et al., 2009; Wilson et al., 2010). Indeed, increased antigenic burden is generally associated with increased T cell responses, and inflammatory signals can promote pathways involved in antigen presentation, T cell proliferation, and T cell survival (Curtsinger and Mescher, 2010; Dresch et al., 2012; van Heijst et al., 2009). While many studies have utilized models of murine infection to elucidate the factors involved in the generation of T cell responses and the formation of memory T cells, vaccination with *cpsII* parasites allows these processes to be studied in a setting in which overt inflammation is absent. Thus, the basic principles learned from studying this system may translate more easily to vaccine design, where inflammation should be limited as well.

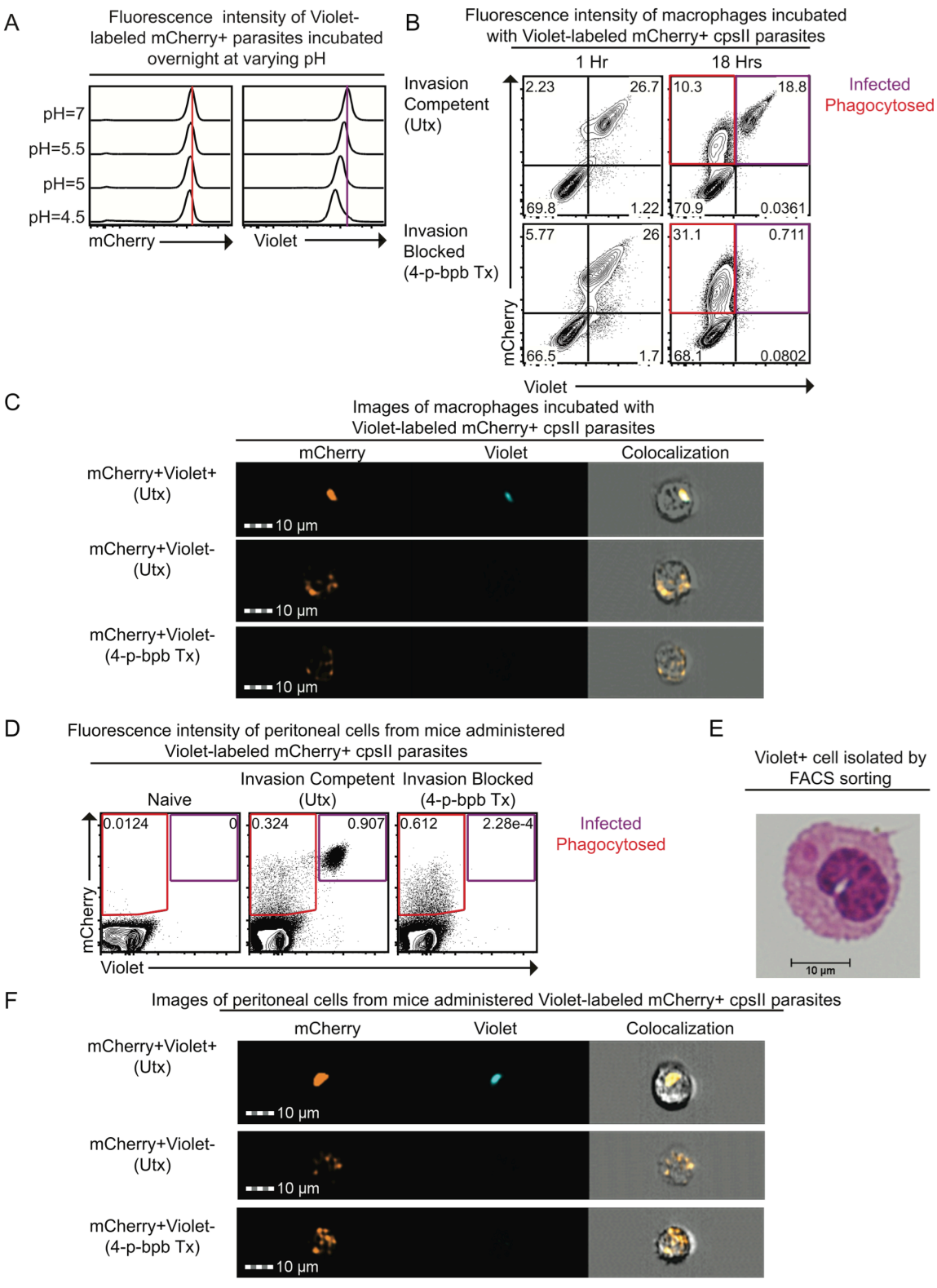

Figure 3.1: Differences in pH sensitivity of two fluorescent markers can be used to distinguish parasites that have been phagocytosed from those that actively invade host

cells. Fluorescence intensity of mCherry-expressing *cpsII* parasites labeled with CellTrace™ Violet and incubated overnight at varying pH in buffer solutions consisting of citric acid and disodium phosphate (McIlvaine, 1921) was measured by flow cytometry (a). Violet and mCherry fluorescence of immortalized murine bone marrow-derived macrophages exposed to Violet-labeled, mCherry-expressing *cpsII* parasites pre-treated with DMSO (top) or the irreversible inhibitor of invasion 4-p-bpb (bottom) 1 hour and 18 hours following exposure to parasites, measured by flow cytometry (b). Images of $mCherry^{+ve}Violet^{+ve}$ and $mCherry^{+ve}Violet^{-ve}$ bone marrow derived macrophages 18 hours following exposure to Violet-labeled, mCherry-expressing *cpsII* parasites pre-treated with 4-p-bpb or DMSO (c). Violet and mCherry fluorescence of cells isolated from the PECS of mice 18 hours post-administration of 10^6 DMSO-treated or 4-p-bpb-treated parasites (d). Cytospin analysis was performed on $Violet^{+ve}$ cells isolated by FACS sorting, obtained from the PECS of a mouse 18 hours after vaccination with Violet-labeled *cpsII* parasites (e). Images of $mCherry^{+ve}Violet^{+ve}$ and $mCherry^{+ve}Violet^{-ve}$ cells isolated from the PECS of mice 18 hours post-administration of 10^6 DMSO-treated or 4-p-bpb-treated Violet-labeled, mCherry-expressing *cpsII* parasites (f).

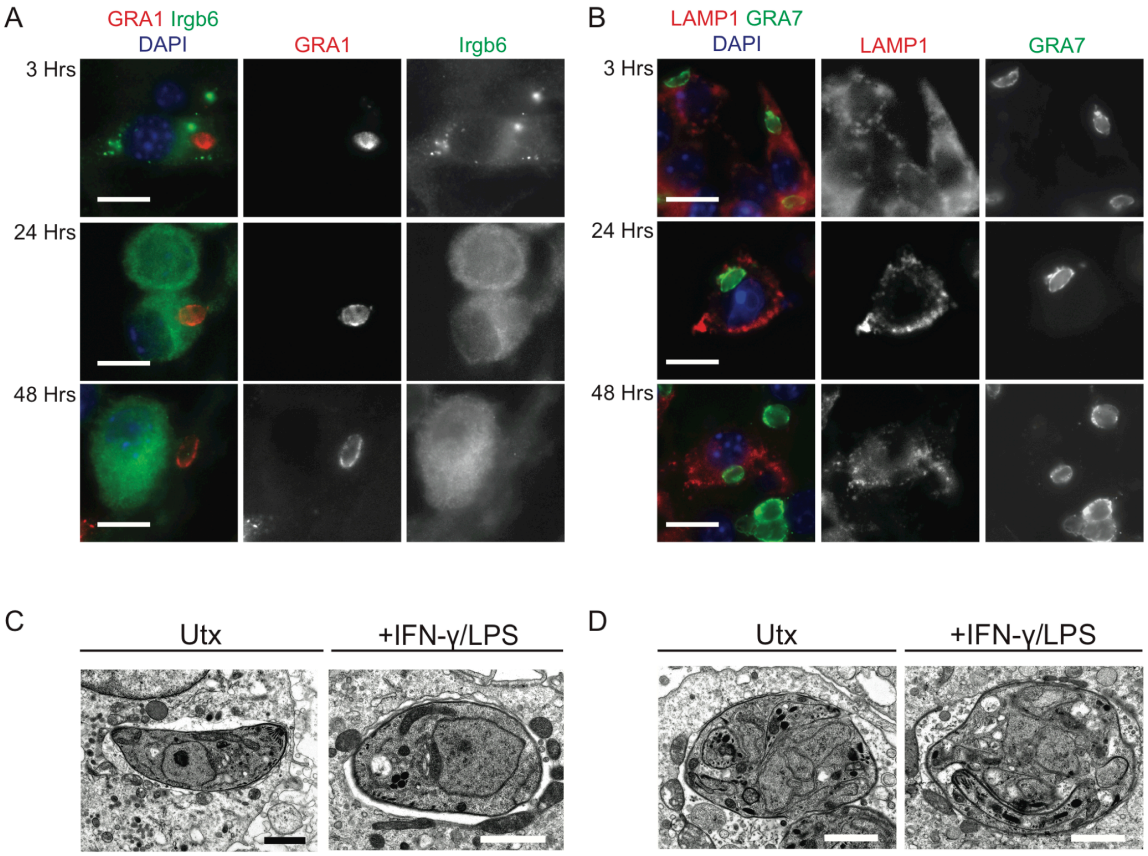

Figure 3.2: The fate of cpsII parasites within infected host cells. RAW macrophages were infected with *cpsII* parasites and examined using immunofluorescence assays (a,b) or electron microscopy (c,d) to determine the fate and cellular localization of the parasites. *CpsII*-infected RAW IFN-γ (100 units/ml) and LPS (0.1 ng/ml) activated-macrophages were stained for GRA1 (a protein secreted by *T. gondii*) to identify the parasitophorous vacuole followed by goat-anti mouse IgG Alexa Fluor 594 and rabbit polyclonal sera against Irgb6 followed by goat anti-rabbit IgG Alexa Fluor 488 (a). LAMP-1 was localized using rat monoclonal antibody 1D4B followed by goat anti-rat IgG Alexa Fluor 594 and the parasite vacuole was localized with rabbit polyclonal sera against GRA7 followed by goat-anti rabbit IgG Alexa fluor 488 (b) at 3 hours, 24 hours and 48 hours post-infection. Scale bars =10um. Electron micrograph images of infected macrophages treated with IFN-γ (50 units/ml) and LPS (10 ng/ml) or untreated at 2 hours post infection (c). Parasites persist in intact vacuoles and do not display blebbing or disruption of the parasitophorous vacuole. Some *cpsII* parasites were found to exhibit non-productive cell division

in IFN-γ and LPS- treated or untreated macrophages when examined 24 hours post-infection (d). Scale bars = 1.5um.

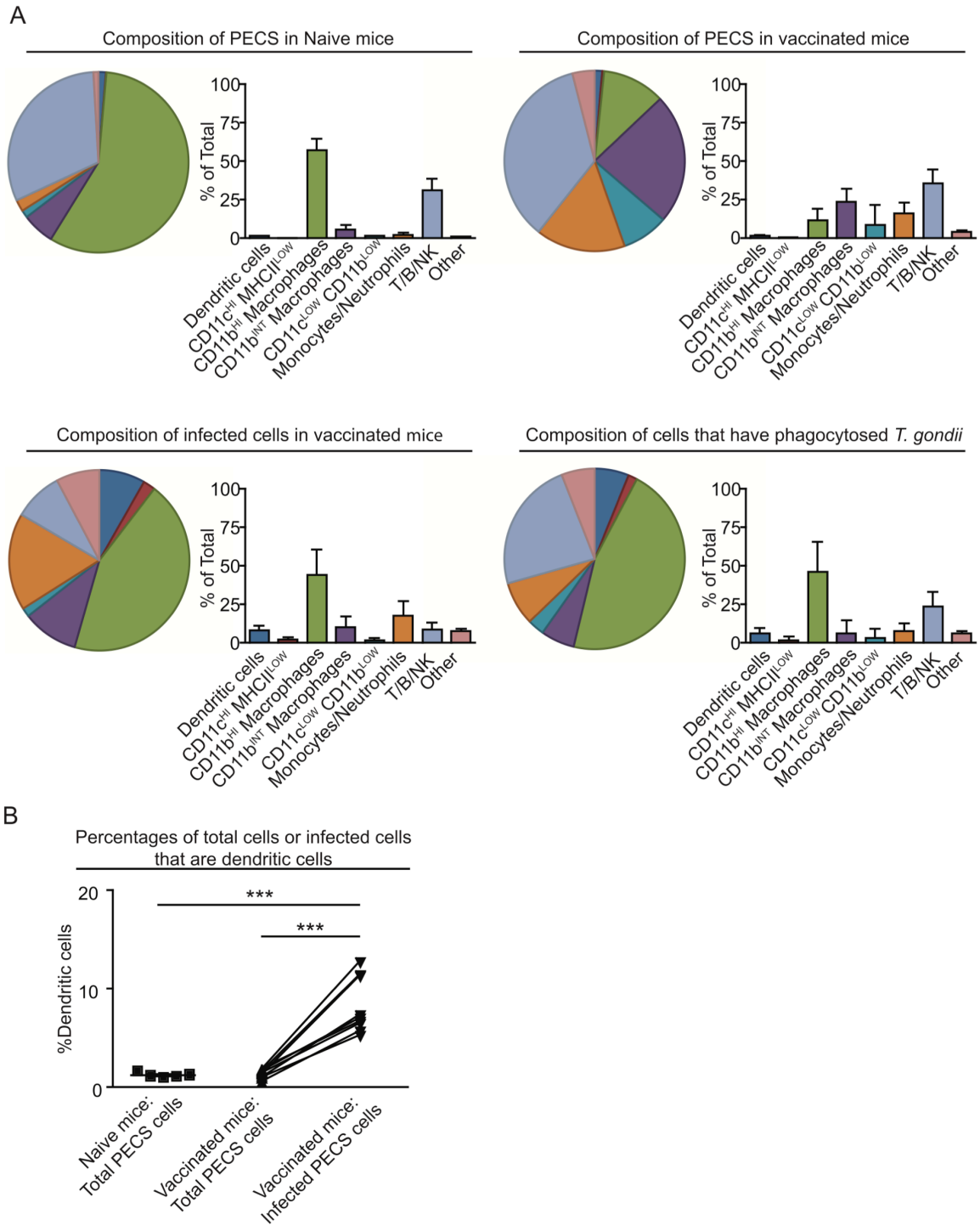

Figure 3.3: Composition of total cell populations, mCherry^{+ve}Violet^{-ve} cell populations, and mCherry^{+ve}Violet^{+ve} populations from the PECS of total mice. Mice were vaccinated with 10^6 Violet-labeled, mCherry-expressing *cpsII* parasites intraperitoneally and sacrificed 18 hours post-vaccination. Cell type composition of total peritoneal cell populations in naïve and vaccinated

mice, and the cell type composition of mCherry^{+ve}Violet^{-ve} cells and mCherry^{+ve}Violet^{+ve} cells in vaccinated mice depicted as pie charts (left) and bar graphs (right) (a). Percentages of Total PECS in naïve mice, total PECS cells in vaccinated mice, and mCherry^{+ve}Violet^{+ve} cells that are dendritic cells are depicted (b). T/B/NK cells are identified by expression of CD3,CD19, or NK1.1. Dendritic cells were identified as CD3^{-ve},CD19^{-ve},NK1.1^{-ve},CD11c^{HI},MHCII^{HI}. Monocytes and neutrophils were defined as CD3^{-ve},CD19^{-ve},NK1.1^{-ve},CD11c$^{LOW-INT}$,Gr-1^{+ve}. Macrophages were identified as CD3^{-ve},CD19^{-ve},NK1.1^{-ve},CD11c$^{LOW-INT}$,Gr-1^{-ve},CD11b^{INTorHI}. *p<0.05;***p<0.0005. AVG±STDEV. A paired, two-tailed student's t test was used to compare the percentages of infected cells that are dendritic cells with the percentages of cells that are dendritic cells in the total PECS of vaccinated mice.

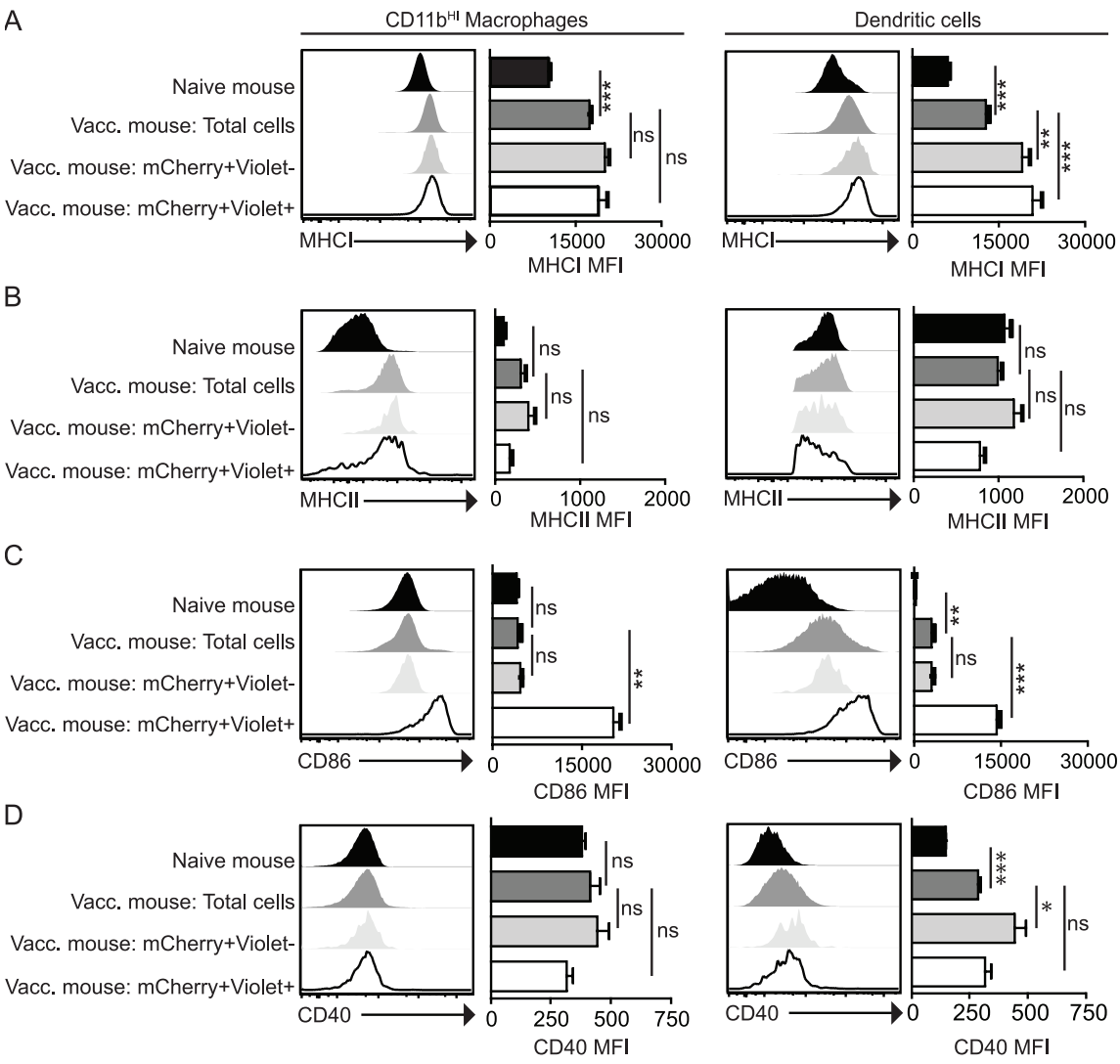

Figure 3.4: Activation status of mCherry^{+ve}Violet^{-ve} and mCherry^{+ve}Violet^{+ve} macrophages and dendritic cells. Mice were administered parasites as described in Figure 3.3. At 18 hours post-vaccination, expression of the antigen presentation molecules MHCI (a) and MHCII (b) and expression of the costimulatory molecules CD86 (c) and CD40 (d) on CD11b^{HI} macrophages and dendritic cells was determined by flow cytometry. Macrophages are identified as CD3^{-ve},CD19^{-ve},NK1.1^{-ve},CD11c^{-ve},Gr-1^{-ve},CD11b^{HI} cells. Dendritic cells are identified as CD3^{-ve},CD19^{-ve},NK1.1^{-ve},CD11c^{HI},MCHII^{HI}. Confidence intervals were determined using the Bonferroni correction method. *p<0.017;**p<0.0017;***p<0.00017. AVG±SE. Paired, two-tailed student's t tests were used to compare expression levels of molecules on populations within *cpsII*-vaccinated mice.

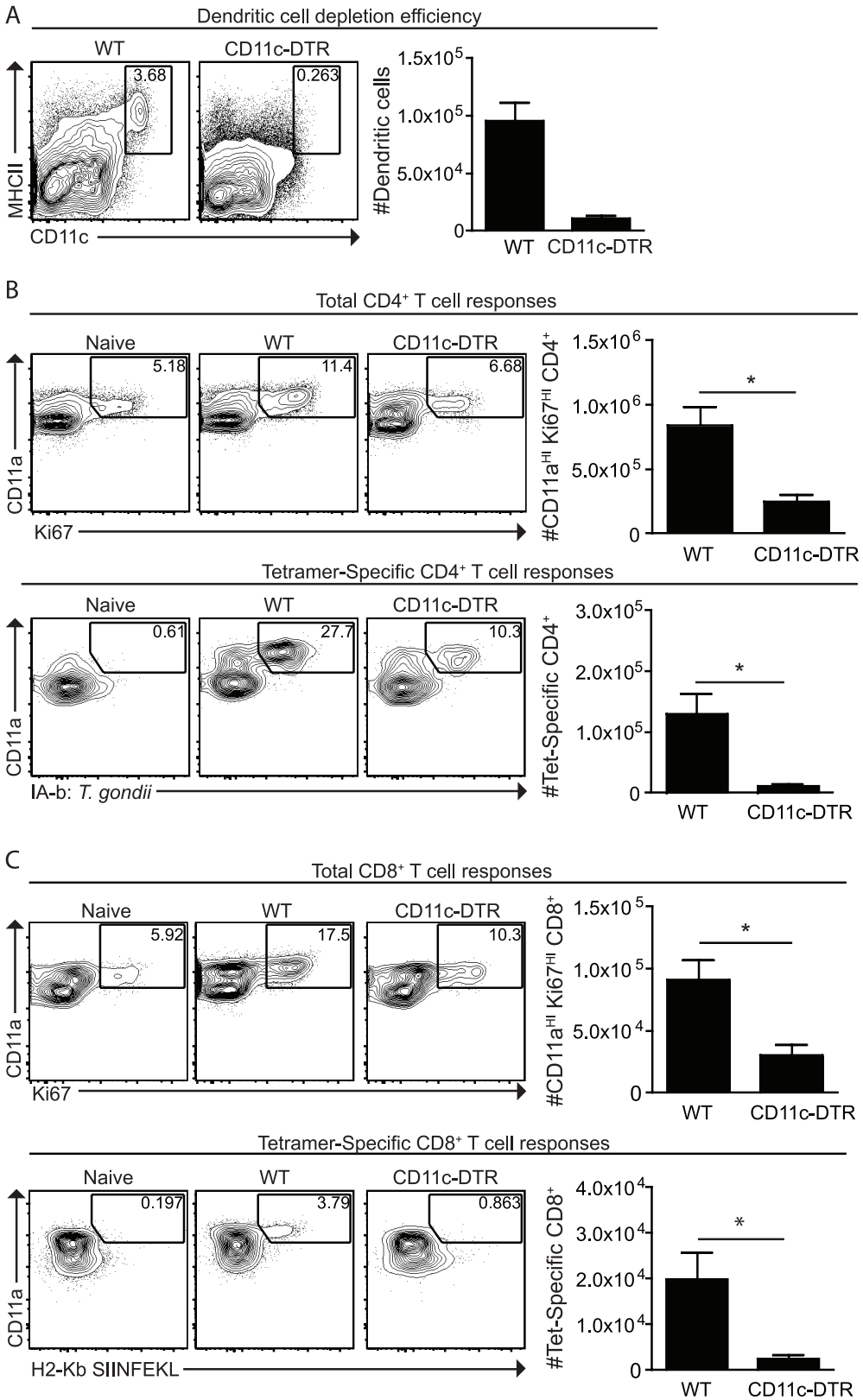

Figure 3.5: Dendritic cells are required for optimal CD4$^+$ and CD8$^+$ T cell responses. CD11c-DTR mice were administered diphtheria toxin 1 day prior to *cpsII*-OVA vaccination. At the time of

vaccination, some mice were sacrificed to determine the efficiency of depletion. Percentages and numbers of dendritic cells from the spleen are shown. FACS plots are gated on $CD3^-$,$CD19^-$,$NK1.1^-$ cells (a). Eight days following vaccination, mice were sacrificed and total and tetramer-specific $CD4^+$ and $CD8^+$ T cell responses were analyzed. Total $CD4^+$ T cell responses from the spleens are shown (b, top). Tetramer-specific $CD4^+$ T cell responses from pooled lymph nodes and splenocytes were determined in a separate experiment (b, bottom). Flow plots are gated on $CD4^+$ T cells (b), and the population examined was magnetically enriched for the $tetramer^{+ve}$ population (bottom). Total and OVA-specific $CD8^+$ T cell responses from the PECS are depicted (c), and flow plots are gated on CD8+ T cells. Significant differences in tetramer and total $CD8^+$ T cell responses between WT and CD11c-DTR mice were also apparent in the spleen. *$p<0.05$; **$p<0.005$. AVG±SE.

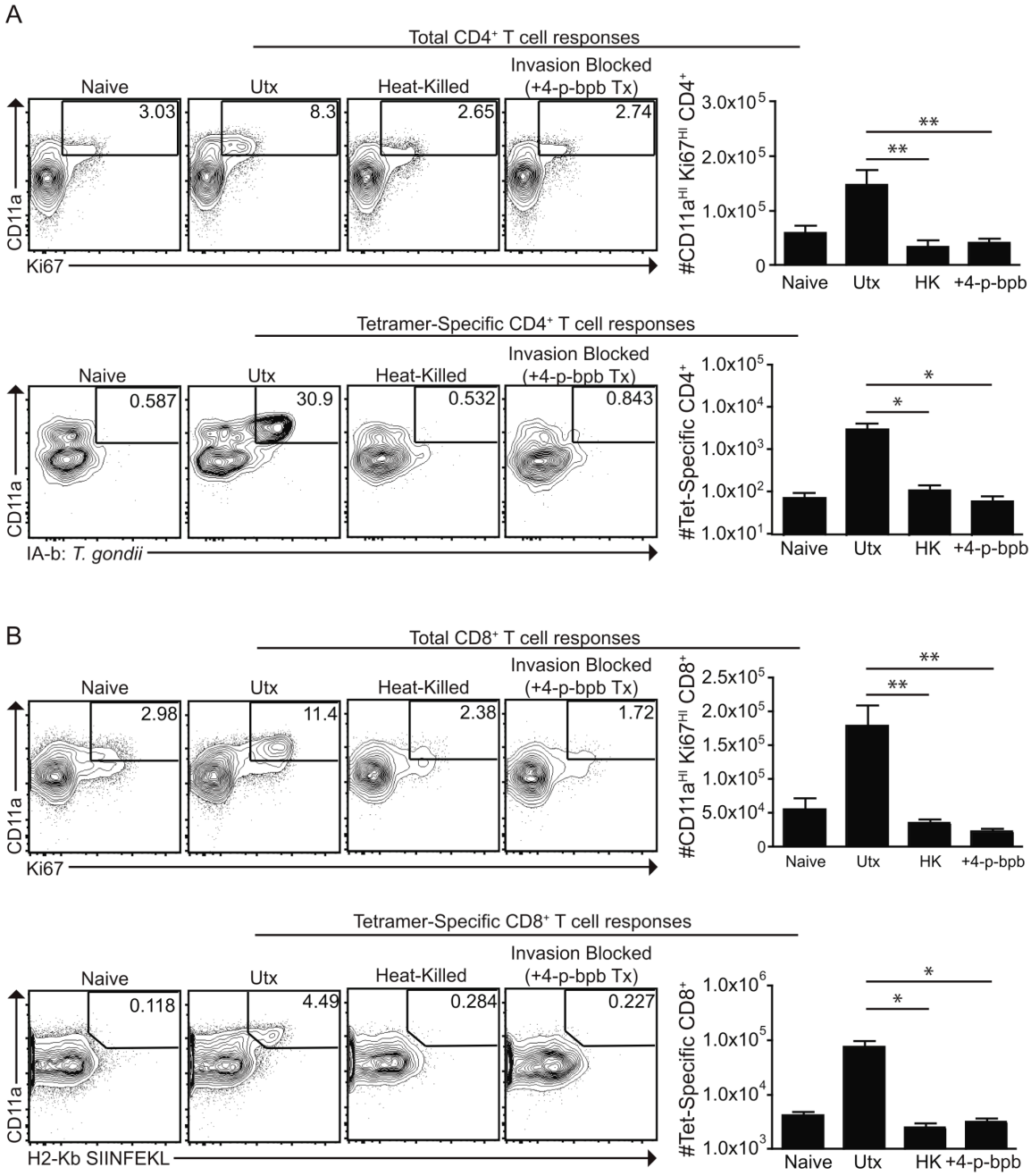

Figure 3.6: Active invasion is required for adaptive immune responses to *T. gondii*. *cpsII*-OVA parasites were heat-killed, treated with the invasion inhibitor 4-p-bpb or left untreated and administered to mice intraperitoneally. Tetramer-specific and total $CD4^+$ (a) and $CD8^+$ (b) T cell responses were measured from cells isolated from the spleen and lymph nodes (pooled) 10 days post-vaccination. Flow plots are gated on $Foxp3^{-ve}$ $CD4^+$ T cells (a, top) or $CD4^+$ T cells (a,

bottom) and the population examined at the bottom of A was enriched for tetramer^{+ve} cells. Flow plots in B are gated on CD8^{+} T cells. *p<0.05; **p<0.005. AVG±SE.

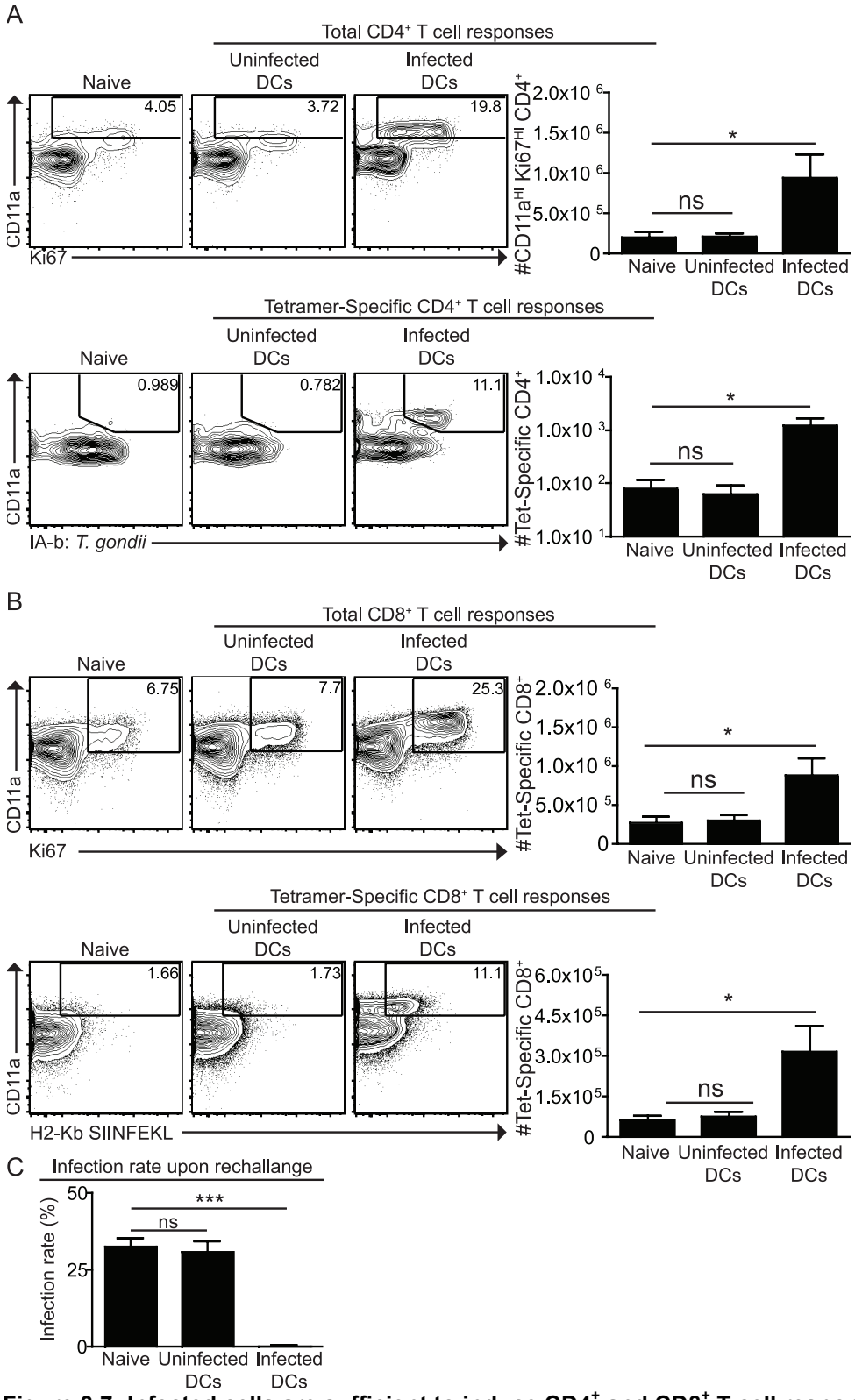

Figure 3.7: Infected cells are sufficient to induce CD4$^+$ and CD8$^+$ T cell responses.

Splenocytes were harvested from WT mice that were administered Flt3L-expressing tumors ~2

weeks prior, magnetically enriched for dendritic cells, and were incubated with Violet-labeled, mCherry-expressing *cpsII*-OVA parasites overnight. The following day, dendritic cells were sorted into mCherry^{+ve}Violet^{+ve} (infected) and mCherry^{+ve}Violet^{-ve} (uninfected) populations and 10^4 dendritic cells from each population were administered to mice. 10 days later, mice were sacrificed and CD4$^+$ (a) and CD8$^+$ (b) T cell responses were analyzed. Pooled splenocytes and lymphocytes are shown. Populations shown depicting CD4$^+$-tetramer binding are enriched for the tetramer^{+ve} population (a, bottom). The data depicting tetramer-specific and total CD4$^+$ T cell responses are from two separate experiments. Flow plots are gated on Foxp3^{-ve} CD4$^+$ T cells (a, top), CD4$^+$ T cells (a, bottom) or CD8$^+$ T cells (b). Parasite burdens from the PECS of mice 5 days post-challenge with 10^3 tachyzoites of a highly virulent, replicating strain of *T. gondii*, administered 21 days following vaccination with 10^4 infected or uninfected dendritic cells, analyzed by flow cytometry (c). *p<0.05; **p<0.005. AVG±SE.

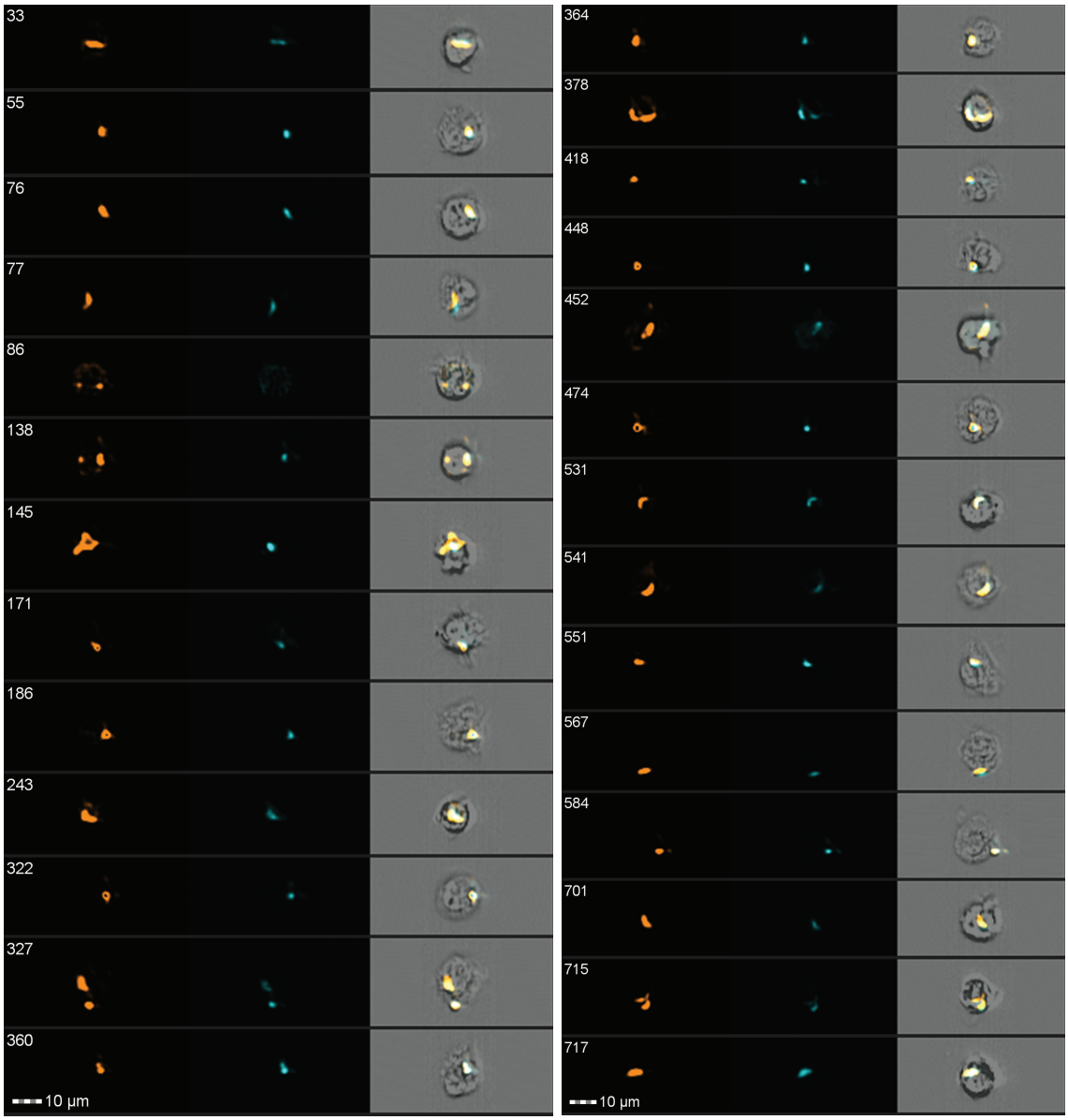

Figure 3.S1: Images of mCherry^{+ve}Violet^{+ve} bone marrow derived macrophages 18 hours following exposure to Violet-labeled, mCherry-expressing *cpsII* parasites.

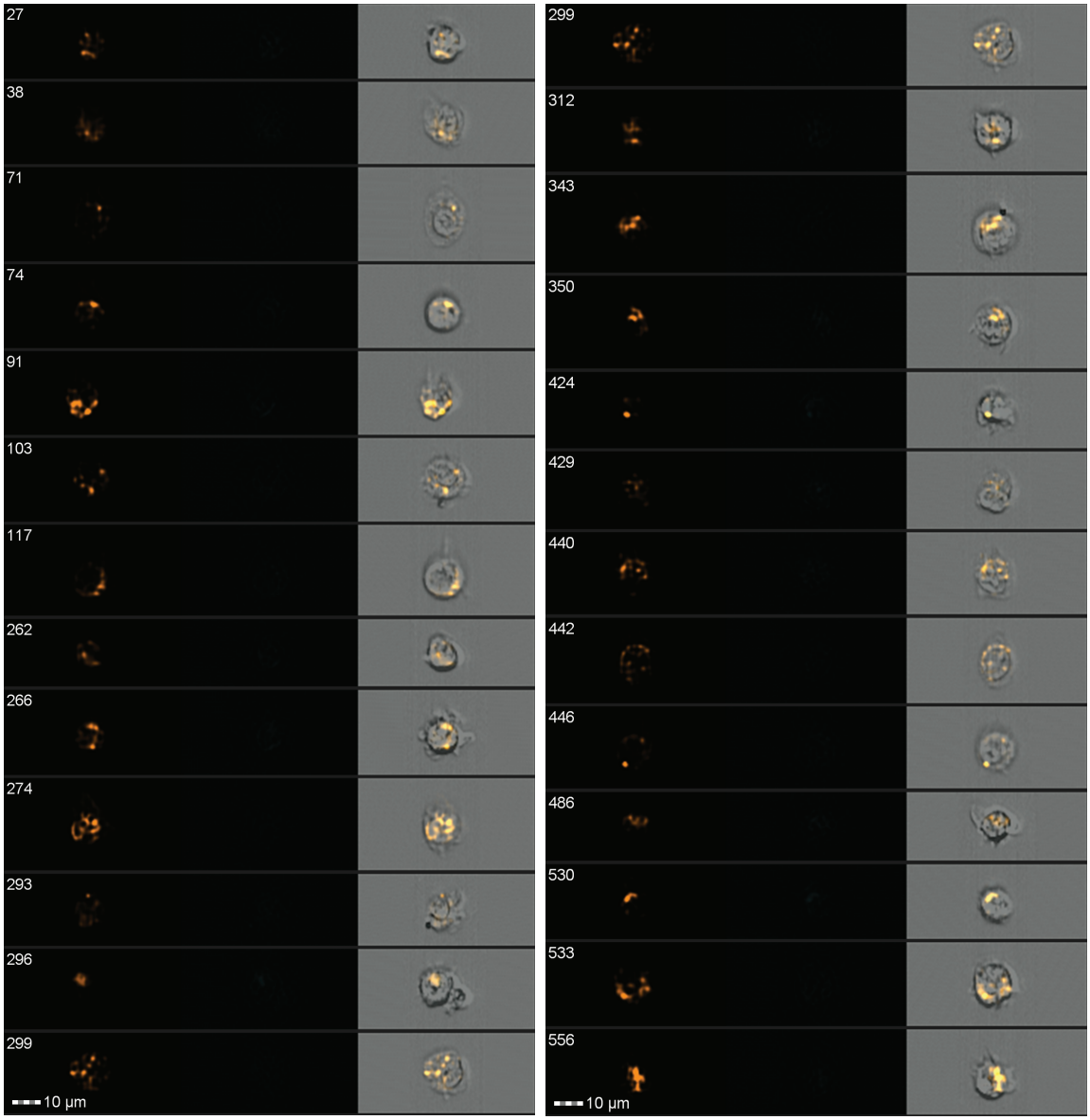

Figure 3.S2: Images of mCherry^{+ve}Violet^{-ve} bone marrow derived macrophages 18 hours following exposure to Violet-labeled, mCherry-expressing *cpsII* parasites.

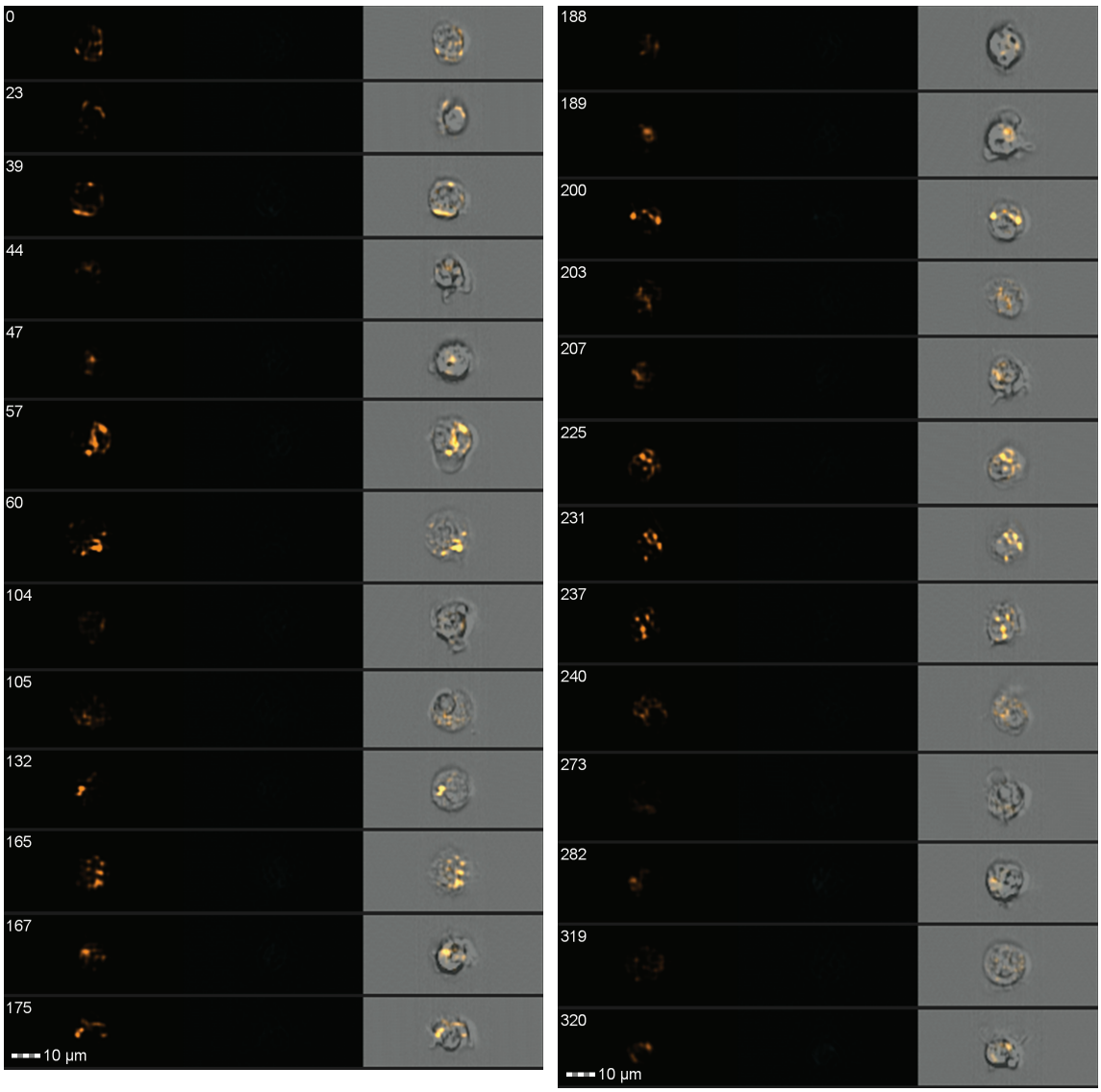

Figure 3.S3: Images of mCherry^{+ve}Violet^{-ve} bone marrow derived macrophages 18 hours following exposure to Violet-labeled, mCherry-expressing *cpsII* parasites, which were pre-treated with 4-p-bpb.

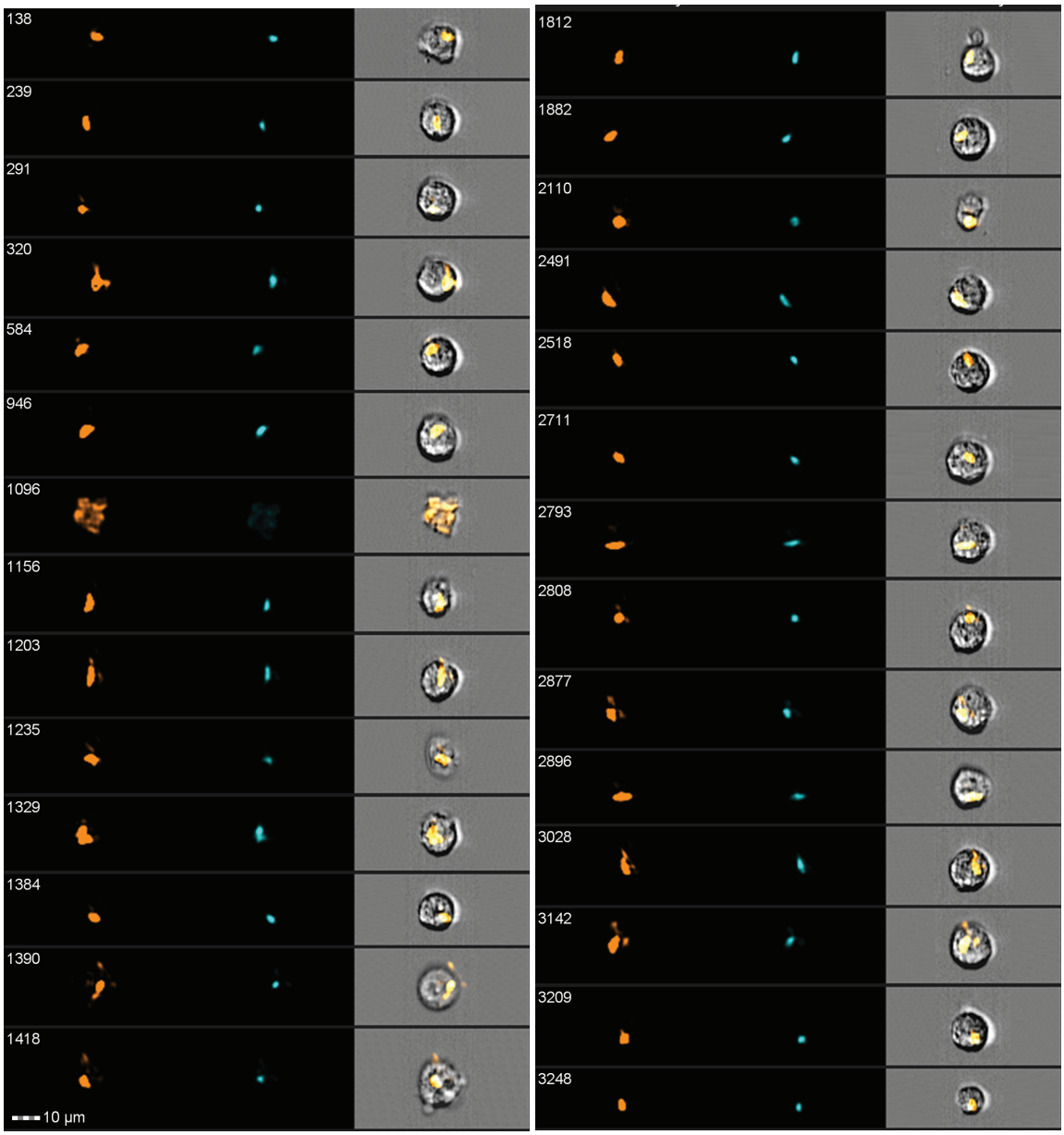

Figure 3.S4: Images of mCherry^{+ve}Violet^{+ve} cells isolated from the PECS of mice 18 hours post-infection with 10^6 Violet-labeled, mCherry-expressing *cpsII* parasites.

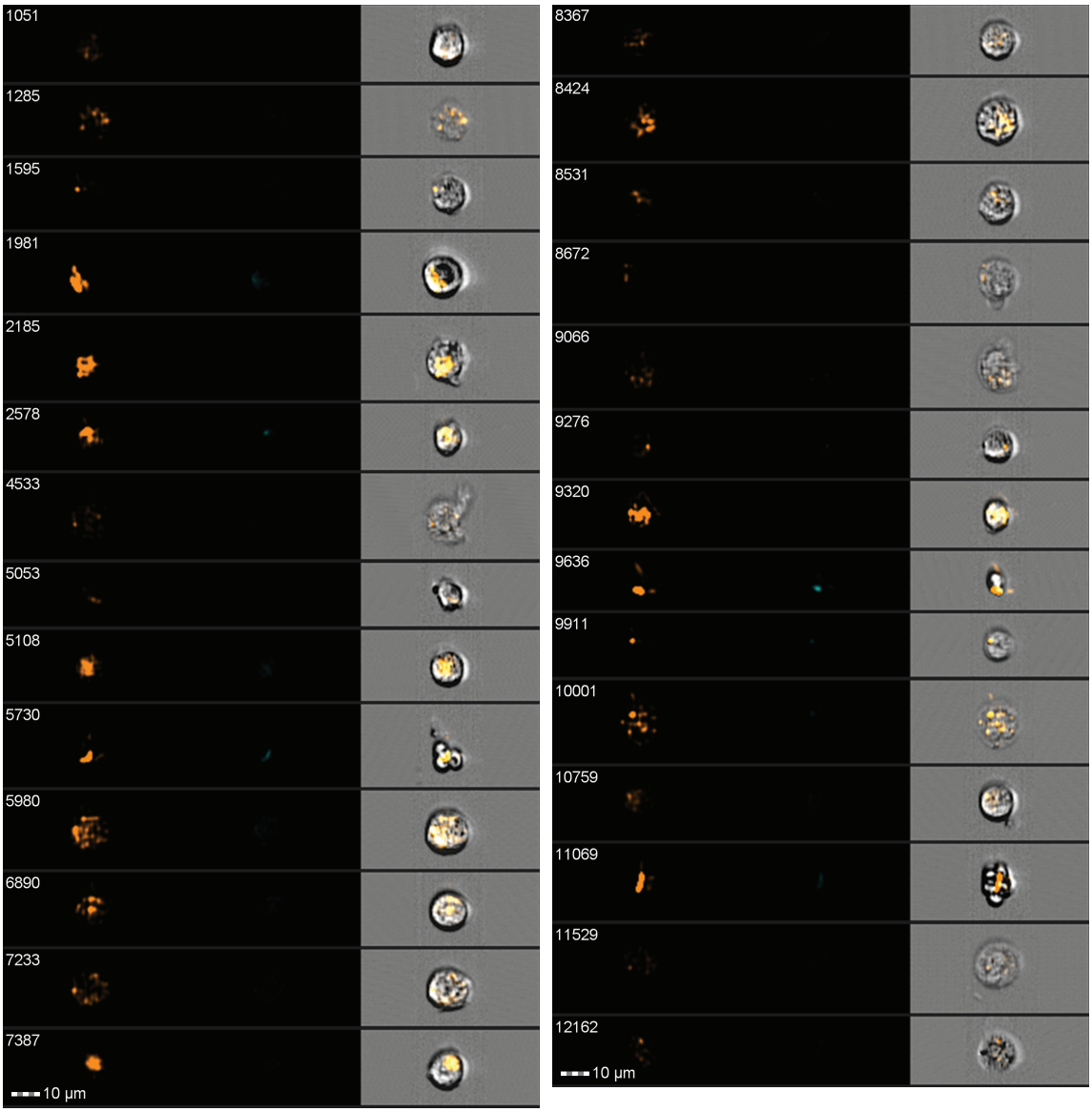

Figure 3.S5: Images of mCherry^{+ve}Violet^{-ve} cells isolated from the PECS of mice 18 hours post-infection with 10^6 Violet-labeled, mCherry-expressing *cpsII* parasites.

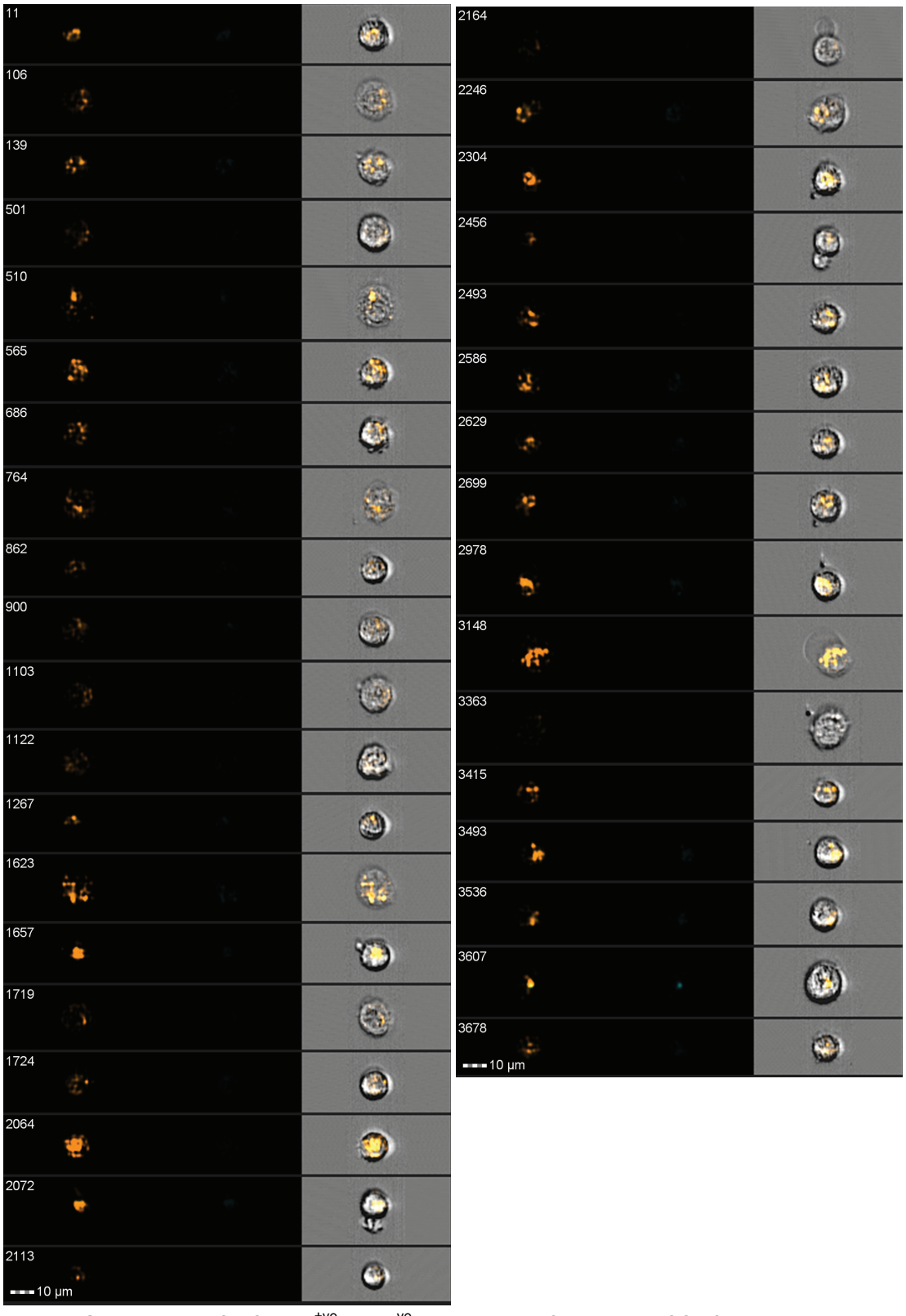

Figure 3S.6: Images of mCherry^{+ve}Violet^{-ve} cells isolated from the PECS of mice 18 hours post-administration of 4-p-bpb-treated, Violet-labeled, mCherry-expressing *cpsII* parasites.

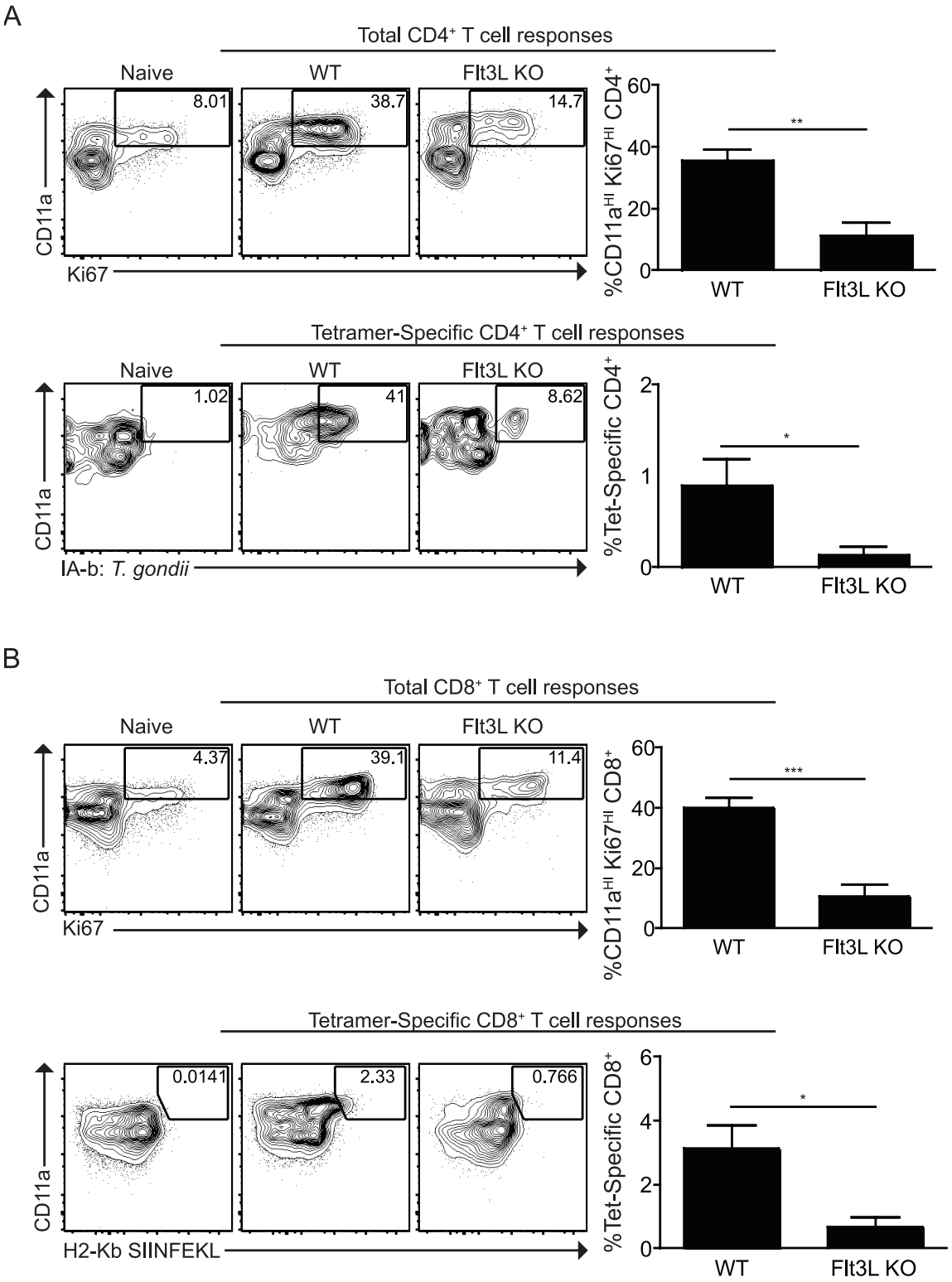

Figure 3S.7: CD4$^+$ and CD8$^+$ T cell responses to *cpsII*-OVA vaccination in Flt3L KO mice.

Flt3L KO mice were vaccinated with 10^5 *cpsII*-OVA parasites intraperitoneally and CD4$^+$ and

$CD8^+$ T cell responses from the spleen and lymph nodes (pooled) were examined at 10 days post-vaccination. $CD4^+$ T cell responses are shown (a). Flow plots shown in A are gated on $Foxp3^{-ve}$ $CD4^+$ T cells (top) or total $CD4^+$ T cells (bottom), and the populations examined at the bottom of A were enriched for the $tetramer^{+ve}$ population. $CD8^+$ T cell responses in the spleen and lymph nodes (pooled) were also examined (b). Flow plots shown in B are gated on $CD8^+$ T cells. *p<0.05; **p<0.005. ***p<0.0005. AVG±SE.

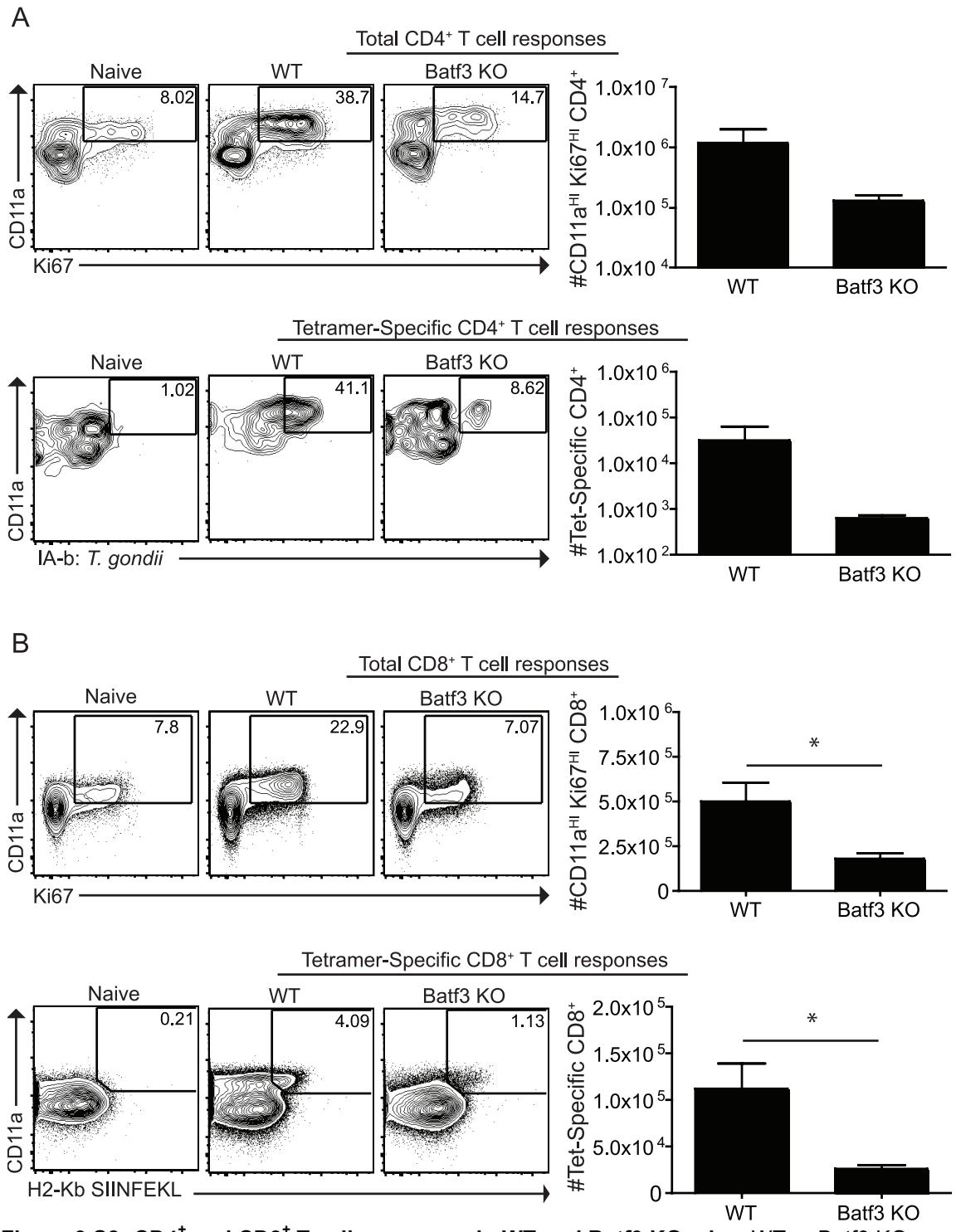

Figure 3.S8: CD4$^+$ and CD8$^+$ T cell responses in WT and Batf3 KO mice. WT or Batf3 KO mice were vaccinated with 10^5 *cpsII*-OVA parasites and examined 10 days post-vaccination. CD4$^+$ T cell responses from the cells isolated from the spleen and lymph nodes (pooled) are shown (a). Flow plots in A are gated on Foxp3^{-ve}CD4+ T cells (top), or total CD4$^+$ T cells (bottom)

and the populations examined in the bottom flow plots are enriched for the tetramer^{+ve} population. $CD8^+$ T cell responses from cells isolated from the spleen and lymph nodes (pooled) are also shown (b). Flow plots in B are gated on $CD8^+$ T cells. *P<0.05. $CD4^+$ and $CD8^+$ T cell data shown are from two separate experiments. AVG±SE.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Introduction

Since their discovery forty years ago, it has become increasingly apparent that dendritic cells serve important roles in bridging the innate and adaptive immune responses (Steinman, 2012). The experiments performed in this thesis, as well as other findings published since these studies were commenced, have underscored the critical role that dendritic cells have in immunity to *T. gondii*. These experiments have also provided novel insight into the mechanisms by which dendritic cell populations are regulated during infection, as well as the specific mechanisms by which dendritic cells must interact with parasites in order to initiate $CD4^+$ and $CD8^+$ T cell responses. In this chapter, the implications of these findings, their applications for human medicine, and future directions for this research will be discussed.

Factors affecting dendritic cell populations during infection

The studies in this thesis examining the role of Flt3L have provided several novel insights regarding the roles of dendritic cells and this cytokine in the immune response to *T. gondii*. In addition to underscoring the important roles that dendritic cells play in immunity, they have also demonstrated that the infection-induced expansion of dendritic cell populations can occur independently of Flt3L. Recent studies have demonstrated that the inflammatory cytokines IL-12 and IFN-γ can induce expression of the transcription factors Batf and Batf2, which can promote development of $CD8\alpha^+$ dendritic cells in the absence of the transcription factor Batf3 (Tussiwand et al., 2012). It is also possible that the IL-12 and IFN-γ-induced expression of these transcription factors contributes to the infection-induced expansion of $CD8\alpha^+$ dendritic cells in wild type mice described herein and described elsewhere (Mashayekhi et al., 2011). This hypothesis could be tested using bone marrow chimeras in which wild type mice are reconstituted with wild type bone marrow mixed with bone marrow deficient for the IL-12 receptor, the IFN-γ receptor, Batf, or Batf2. The infection-induced increase in numbers of dendritic cells may also be partially accounted for by the conversion of inflammatory monocytes to dendritic cells. Indeed, recent studies have demonstrated that adoptively transferred congenic inflammatory monocytes can

develop into dendritic cells in mice infected with *T. gondii* (Goldszmid et al., 2012). It is also important to note that while the studies described herein demonstrate that the expansion of dendritic cells can occur independently of Flt3L, this does not rule out the possibility that Flt3L may have a partial or redundant role in this process. Neutralizing Flt3L or the cytokine GM-CSF (which promotes the development of inflammatory monocytes into dendritic cells (Xu et al., 2007)) in wild type mice during infection with *T. gondii* may be effective strategies to dissect the role that these cytokines play during infection from their roles in dendritic cell homeostasis.

The physiological implications of the inflammation-induced expansion of dendritic cells

While the mechanisms by which the infection-induced expansion of dendritic cells occurs remain unclear, previous studies have provided insight into the potential physiological significance of this phenomenon. For example, studies comparing the $CD8^+$ T cell responses during infection with the highly virulent RH strain of *T. gondii* with the $CD8^+$ T cell responses induced by the less virulent Pru strain revealed that a greater expansion of dendritic cells during infection with Pru correlated with greater $CD8^+$ T cell responses (Tait et al., 2010). Similarly, an infection-induced increase in numbers of dendritic cells during infection with *Plasmodium* resulted in greater $CD8^+$ T cell responses (Guermonprez et al., 2013). In contrast to these reports, vaccination with the *cpsII* strain of *T. gondii* was found to induce $CD8^+$ T cell responses of similar magnitude to the Pru strain, despite the fact that no expansion of dendritic cells was apparent following vaccination with *cpsII* (Jordan et al., 2009)(unpublished data). One possible explanation for these findings is that dendritic cell expansion is critical for immunity in the context of infection, but not vaccination. Comparisons between infection with replicating and replication-deficient strains of *T. gondii* may provide a convenient system for interrogating the differential requirements for dendritic cell expansion, and identifying the factors that govern this expansion during toxoplasmosis will also be helpful in determining the physiological significance of this phenomenon.

Infection-induced expression of Flt3L

Given that ligands for TLRs 3,4 and 9 can induce increased expression of Flt3L in vivo (Eidenschenk et al., 2010), and *T. gondii* can signal through TLR4 (Debierre-Grockiego et al., 2007), it was somewhat surprising that expression of Flt3L was not increased following infection with *T. gondii*. This finding also contrasts with work showing that increased expression of Flt3L is induced following infection with Plasmodium and Mouse Cytomegalovirus (Debierre-Grockiego et al., 2007; Guermonprez et al., 2013). Interestingly, the *Plasmodium*-induced expression of Flt3L was dependent upon Type I Interferon (IFN) responses (Guermonprez et al., 2013), and while much is known about the ability of *T. gondii* to drive production of IL-12 and IFN-γ, relatively less is known regarding the role of Type I IFNs during toxoplasmosis, or the parasite's ability to induce these responses (Lieberman and Hunter, 2002). Perhaps Type I IFN-induced expression of Flt3L and IL-12 driven expression of Batf and Batf2 (discussed in the previous section) are parallel pathways by which hosts can expand dendritic cell populations during different types of infections.

Implications for human disease

While the increased susceptibility of Flt3L KO mice to toxoplasmosis underscores the importance of Flt3L in immunity, identifying therapeutic applications for Flt3L is confounded by the pleiotropic properties of this cytokine. Thus, while Flt3L promotes optimal immune responses against infectious pathogens (Eidenschenk et al., 2010; Guermonprez et al., 2013), and has been used as an adjuvant to enhance responses to vaccination or immunotherapy (Kodama et al., 2010; Mwangi et al., 2011; Mwangi et al., 2002; Song et al., 2009; Yo et al., 2007; Zhou et al., 2010; Zurkova et al., 2009), treatment of mice with Flt3L can also result in increased numbers of regulatory T cells that inhibit auto-immune disease (Darrasse-Jeze et al., 2009). It may therefore be important to identify the variables that determine whether Flt3L promotes immune responses or inhibits them, in order to predict the effects of treating patients of various diseases with this cytokine. Given that toxoplasmosis is a disease that requires both an adequate Th1 immune response to control infection, and anti-inflammatory mechanisms to prevent severe

immunopathology (discussed in Chapter 1), murine infection with *T. gondii* may prove to be a useful model to further interrogate the roles of this cytokine.

The role of infected cells in generating CD4+ and CD8+ T cell responses

The studies in this thesis demonstrated that actively infected host cells, rather than cells that phagocytose *T. gondii* parasites, are potent inducers of $CD4^+$ and $CD8^+$ T cell responses. Several possible explanations may account for this. One possibility is that active invasion allows parasites to persist for longer periods than when they are phagocytosed and rapidly degraded, and this persistence of antigen is necessary to generate optimal immune responses. This idea stands in contrast to results obtained from studies using an attenuated strain of *Listeria*, in which T cell responses were similar regardless of the when the bacteria was cleared by treatment with antibiotics (Mercado et al., 2000). Another possibility is that the persistence of parasites results in greater quantities of antigen, since many antigens from *T. gondii* that are recognized by $CD4^+$ and $CD8^+$ T cells are secreted by the parasite (Blanchard et al., 2008; Frickel et al., 2008; Gregg et al., 2011; Kwok et al., 2003; Pepper et al., 2004). However, CD8+ T cells specific for the protein Tgd_057, which is expressed in the cytoplasm of the parasite (Wilson et al., 2010), were also abrogated when invasion was blocked, suggesting that increased amounts of secreted antigen can not solely account for the critical role for invasion to generate $CD4^+$ and $CD8^+$ T cell responses.

Another possible explanation for the failure of phagocytosed parasites to induce $CD4^+$ or $CD8^+$ T cell responses is that this pathway may provide insufficient levels of inflammatory signals, and the failure of cells that have phagocytosed *T. gondii* to upregulate the co-stimulatory molecule CD86 is consistent with this idea. Inflammatory stimuli can be required to promote phagosomal maturation and antigen processing (Blander, 2008; Blander and Medzhitov, 2006), or to induce the expression of cytokines and co-stimulatory signals necessary for T cell activation (Chen and Flies, 2013; Joffre et al., 2009), and several factors may account for the ability of live (as opposed to phagocytosed) parasites to uniquely activate innate sensing mechanisms. Since some antigens from *T. gondii* that are sensed by innate immune receptors may be secreted (such

as profilin (Pifer et al., 2011)), the increased persistence of parasites that occurs when they are able to invade host cells may result in greater amounts of inflammatory stimuli. The distribution of innate immune receptors within host cells also may result in the activation of unique pathways, depending on whether parasites are phagocytosed or actively invade (Iwasaki and Medzhitov, 2010). Lastly, metabolic products from live parasites may activate sensing mechanisms that are not stimulated by dead (and thus non-metabolically active) parasites. The specific host factors that may be involved in the sensing of *cpsII* parasites are discussed in greater length in a later section of this chapter.

Pathways that may lead to antigen presentation following cpsII vaccination

Regardless of the underlying cause of the requirement for active invasion to generate T cell responses, this requirement suggests that either infected cells directly present antigen to T cells, or that infected cells are phagocytosed by cells (a process referred to as efferocytosis), and the efferocytosing-cell then primes naive T cells (Figure 4.1). Previous studies have been interpreted to support the idea that infected cells directly prime naïve T cells (Dzierszinski et al., 2007; Goldszmid et al., 2009; Gubbels et al., 2005), however it must be remembered that some of these studies did not distinguish active invasion from phagocytosis with the same granularity as the studies performed in this thesis, and many of the past studies utilized in vitro systems, which may not reflect what occurs in vivo. In vivo imaging studies have observed dendritic cells that do not contain intact parasites to interact extensively with antigen-specific T cells, suggesting that uninfected cells prime T cells (John et al., 2009) (Chtanova et al., 2009). Consistent with the idea that antigen is acquired through the phagocytosis of infected cells (i.e. efferocytosis), previous studies have demonstrated that antigens derived from transferred murine dendritic cells can be expressed in the context of MHC molecules on the dendritic cell population of recipient mice (Inaba et al., 1998), and expression of MHCII is required from recipient mice for optimal $CD4^+$ T cell responses following the transfer of peptide-pulsed dendritic cells (Kleindienst and Brocker, 2003). In the following sections, the implications of these two models are discussed.

Implications of the direct presentation of antigen by *cpsII*-infected cells to prime naïve T cells

One surprising aspect of the model that infected cells directly prime $CD4^+$ and $CD8^+$ T cell responses is that the parental strain of the *cpsII* parasites (the RH strain) is known to induce an alternatively-activated phenotype in the cells that in infects, characterized by expression of the enzyme arginase-1, and reduced expression of IL-12 (Butcher et al., 2011; Saeij et al., 2007). Unlike classically activated macrophages, alternatively activated macrophages promote immune responses to allergens and helminthes, participate in anti-inflammatory responses, and are thought to be poor inducers of Th1 immune responses (Varin and Gordon, 2009). While the studies described in this thesis demonstrated that the infected cells were activated, they did not distinguish classical activation from alternative activation. Further experimentation will be required to determine whether *cpsII* parasites, like RH parasites, induce alternative activation, and to identify the sources of IL-12 that drive the $CD8^+$ T cell response following vaccination with c*psII* parasites. The dual-fluorescence reporter system developed in this thesis, used in combination with intracellular cytokine staining or fluorescent reporter systems to measure IL-12 production (Reinhardt et al., 2006), may help to answer this question.

Implications of efferocytosis as a means of acquiring antigen for presentation following *cpsII* vaccination

The hypothesis that infected cells are phagocytosed implies a mechanisms by which these cells can be identified as targets for efferocytosis. In current models, cells are identified as targets for phagocytosis when they undergo apoptosis, and express phosphatidylserine on their surface, which ligates receptors on the surface of the efferocytosing-cell. This suggests the possibility of an innate sensing mechanism by which infected cells induce their own cell death in order to become targets for efferocytosis, and studies using bacterial models of infection have provided precedence for infection-induced apoptosis (Philip and Brodsky, 2012) as well as the efferocytosis of infected cells (Martin et al., 2012). However, many studies have observed *T. gondii* to have anti-apoptotic effects on infected cells (Laliberte and Carruthers, 2008). Perhaps these anti-apoptotic effects are impaired in *cpsII* parasites, as a result of their Uracil auxotrophy.

Alternatively, a direct sensing mechanism and infection-induced apoptosis may be unnecessary for efferocytosis to occur, as the relatively short half life of dendritic cells in vivo (Liu and Nussenzweig, 2010) may cause a sufficient number to be efferocytosed regardless of their infection status.

Another implication of the efferocytosis model is that the efferocytosing-cell must present the antigens it phagocytoses to naïve T cells that develop into effector cells, however this idea stands in contrast to current models that have emphasized the role of efferocytosis in inducing tolerogenic T cell responses (Steinman et al., 2003). Thus, if the efferocytosis model is correct, there is likely a sensing mechanism that leads the efferocytosing-cell to provide stimuli that promote Th1, rather than tolerogenic T cell responses. Such a sensing mechanism could conceivably result from the direct ligation of innate immune receptors in the efferocytosing-cell by foreign antigen, or from inflammatory mediators released by the infected cell that act upon the efferocytosing-cell (Figure 4.2). Regardless, determining the fate of infected cells *in vivo* will help to determine if antigen is in fact acquired through efferocytosis, and experiments to interrogate the relative contributions of efferocytosis and direct priming by infected cells are discussed in the following section.

Future applications of the adoptive transfer of *cpsII*-infected dendritic cells

The adoptive transfer system described in this thesis may be useful to determine the fate of infected cells, and this information could help to determine whether antigen is acquired through efferocytosis. Thus, infected congenic cells could be adoptively transferred to mice, and monitored over time to determine if they clear parasites, persist for long periods of time, or migrate to other locations. The dual-fluorescence system utilized in this thesis to distinguish active invasion from phagocytosis could easily be modified to detect efferocytosis in vivo, by engineering cells that express mCherry, infecting them with parasites, and labeling the infected cells with CellTrace™ Violet. This system could also be used to characterize the cells responsible for efferocytosis. Monitoring the interactions between infected cells and naïve T cells, or potential

efferocytosing-cells by two-photon microscopy may also provide insights into the mechanisms by which antigen is acquired for presentation.

The adoptive transfer of infected dendritic cells also provides a convenient system to distinguish the factors that are required from infected cells to generate $CD4^+$ and $CD8^+$ T cell responses from those that are required from uninfected cells. For example, while IL-12 has been implicated in promoting $CD8^+$ T cell responses following *cpsII* vaccination (Wilson et al., 2010; Wilson and Hunter, 2008), it is currently unclear which cells (infected, or uninfected) are critical sources of this cytokine. The role of infected cells as sources of IL-12 could be directly tested by transferring pre-infected IL-12p40-deficient cells to wild type mice, and comparing the resulting $CD4^+$ and $CD8^+$ T cell responses to those induced by the transfer of pre-infected wild type dendritic cells. Similarly, the roles of infected cells in direct presentation of antigen could be interrogated by transferring dendritic cells deficient in MHCI or MHCII, and comparing the resulting $CD4^+$ and $CD8^+$ T cell responses to those induced by the transfer of wild type dendritic cells. Manipulating dendritic cells prior to transfer could also be useful to test whether their efferocytosis leads to antigen presentation. Thus, transferred pre-infected dendritic cells could be treated with inhibitors of apoptosis, agents that promote apoptosis, factors that block efferocytic uptake (such as Annexin V, which binds phosphatidylserine (Fadok et al., 1998)) or factors that promote efferocytosis (such as anti-CD47 blocking antibodies or opsonizing antibodies for mouse cells (Gardai et al., 2006)) to determine the role that efferocytosis has in generating $CD4^+$ and $CD8^+$ T cell responses.

The use of mCherry to study intracellular trafficking

Regardless of whether antigen is directly presented by infected cells or presented by cells that phagocytose infected cells, the intracellular pathways by which phagocytosed antigens are processed and ultimately expressed on the surface of host cells remain to be determined. The ability of mCherry fluorescence to persist long after parasites have been degraded may enable the intracellular trafficking pathways by which phagocytosed antigens are processed to be tracked. Phagocytosed mCherry-expressing parasites may therefore be useful to determine how

T. gondii antigen is ultimately presented, and why cells that phagocytose free parasites fail to present antigen. This technique may be broadly applicable to studying antigen presentation and intracellular trafficking pathways in a variety of systems, provided that the targets for phagocytosis can be labeled with mCherry. Combining this technique with other methods to fluorescently label intracellular structures (such as the use of LysoTracker® to identify acidified organelles or the use of cells expressing GFP-labeled MHCII molecules (Haller et al., 1996) (Chow et al., 2002)) may prove to be valuable in examining intracellular trafficking pathways. Such studies may provide clarity into the mechanisms by which phagocytosed antigens are cross-presented, which is currently an area of active research (Dresch et al., 2012; Joffre et al., 2012; Lin et al., 2008).

Innate sensing of *Toxoplasma gondii*

While many reports have examined the roles of TLRs in immunity to *T. gondii* (Miller et al., 2009) (Denkers, 2010; Egan et al., 2009b; Pifer and Yarovinsky, 2011), fewer studies have examined the roles of TLR-independent mechanisms of sensing *T. gondii*, and there are several reasons to think these pathways are important. Firstly, the signaling molecule MyD88, which is required to mediate downstream signaling pathways from most TLRs, is not necessary for the generation of *cpsII*-induced immunity to *T. gondii* (Sukhumavasi et al., 2008). Secondly, a report published since the time these studies commenced observed the upregulation of CD86 in infected cells to occur independently of MyD88 (Morgado et al., 2011). Additionally, the unique phenotypes of cells that have phagocytosed *T. gondii* from those that are infected by the parasite point toward distinct sensing mechanisms for phagocytosed parasites, and those that actively invade host cells. Whereas TLRs are generally thought to sense phagocytosed antigens, the sensing of foreign pathogens within infected host cells is thought to be mediated by cytosolic receptors (such as the Nod-like receptors) (Iwasaki and Medzhitov, 2010). Collectively, these data point toward a role for TLR-independent mechanisms in sensing *T. gondii* and initiating adaptive immune responses. These results stand in contrast to current models that emphasize the roles of TLR-stimulation in promoting antigen presentation (Blander, 2008). Identifying the innate mechanisms

that lead to the sensing of *cpsII* parasites and the presentation of parasite antigens following vaccination may therefore allow these pathways to serve as targets for vaccine design.

Limitations of the *cpsII* model

While *cpsII* parasites have proven to be a useful tool to understand the host immune response to *T. gondii*, care must be taken in extrapolating the findings from these studies to a natural infection characterized by parasite replication, lysis of infected cells and overt inflammation. Inflammation can promote cross presentation (Dresch et al., 2012) and it is possible that other pathways of antigen presentation are engaged in this setting. However, IFN-γ has microbiostatic effects on *T. gondii* (Langermans et al., 1992), which may recapitulate the pathways seen following *cpsII* vaccination during infection with replicating parasites. Regardless, the principles learned from studying systems in which overt inflammation is absent may be more applicable to the design of vaccines, which ideally should not induce as much inflammation as acute infection.

Phagocytosis of *T. gondii* parasites in vivo

The finding that parasites can be phagocytosed following infection raises the question of how the phagocytosis of parasites occurs in vivo. *T. gondii*-specific and non specific antibodies, as well as complement have been implicated in the opsonization of *T. gondii* for phagocytosis (Johnson et al., 1996) (Hammouda et al., 1995a; Schreiber and Feldman, 1980; Suzuki and Tsunematsu, 1971; Vercammen et al., 1999). Furthermore, antibody-mediated opsonization may be critical for immunity to *T. gondii*, as mice deficient in B cells are susceptible to toxoplasmosis, and the passive transfer of anti-*T. gondii* antibodies can confer protection (Kang et al., 2000). The use of dual fluorescent reporters with varying pH sensitivity, and pharmacological inhibitors may be a useful system to address the roles of various factors in promoting phagocytosis in vivo. In addition to providing insight regarding the roles of these factors in immunity to *T. gondii*, the dual florescent reporter system may aid in testing the role of phagocytosis in immunity to other pathogens, or in determining the fate of various therapeutic agents in vivo.

Acidification of dendritic cell phagosomes

One implication of the loss of violet fluorescence observed when dendritic cells phagocytose Violet-labeled, mCherry expressing parasites is that dendritic cell phagosomes acidify when parasites are phagocytosed. While some studies have observed the phagosomes of dendritic cells to acidify (reaching pH values as low as 4.5) (Bettiol et al., 2010; Erwig et al., 2006; Thiele et al., 2003; Trombetta et al., 2003), other studies have observed dendritic cell phagosomes maintain a pH of ~7.5 following phagocytosis (Mantegazza et al., 2012; Savina et al., 2006). This apparent discrepancy may result from kinetic changes in the phagosomal pH over time, as the studies performed in this thesis examined 18 hours after exposure to parasites, and the more neutral pH has been observed within three hours of phagocytosis. Another potential explanation is that phagosomal maturation is governed by inflammatory signals, and the inflammatory signals provided by *T. gondii* differ than those provided by latex beads or LPS treatment of cells. Indeed, TLR signaling can increase rates of phagocytosis and phagosomal maturation in macrophages (Blander, 2008), and *T. gondii* has been reported to signal through TLRs 2,4,11 and 12 (Debierre-Grockiego et al., 2007; Koblansky et al., 2013; Yarovinsky et al., 2005). The use of invasion-blocked parasites and the mCherry/CellTrace^{TM} Violet dual fluorescence system may therefore be a useful tool to answer questions regarding phagosomal maturation in a more physiological setting than occurs when latex beads are phagocytosed. Insights gained using such a system may prove useful for the optimization and design of vaccines or therapeutics that target the phagocytic pathway.

Towards therapeutic applications of cpsII parasites

Several aspects of the *cpsII* strain suggest the potential of replication-deficient *T. gondii* parasites to be used as a vaccine for toxoplasmosis, or as a vector for the delivery of immunotherapeutic antigens or antigens for vaccination against other pathogens. These qualities include the ability of the *cpsII* strain to induce long-lived $CD4^+$ and $CD8^+$ T cell responses, as well as its ability to preferentially invade dendritic cells. Additionally, these parasites can be easily cultured in the laboratory setting, unlike many other pathogens. In support of this idea, another attenuated strain

of *T. gondii* (the Ts-4 strain) has been used as a vector for vaccination against *Plasmodium yoelii*, and conferred partial protection from challenge with this pathogen (Charest et al., 2000). However, the *cpsII* strain has been observed to occasionally revert to its wild-type phenotype, resulting in the death of vaccinated mice (data not shown), and therefore is not sufficiently safe for therapeutic use in humans. Furthermore, while *cpsII* vaccination can induce memory responses that protect against challenge with *T. gondii*, protection against challenge with other pathogens may require quantitatively or qualitatively different T cell responses. For example, full protection against *Plasmodium* may require extremely large pools of memory T cells (Schmidt et al., 2010), which are much larger than those induced by *cpsII* vaccination. Further genetic manipulation of the parasite may therefore be necessary to make attenuated *T. gondii* sufficiently safe for use in humans, and to optimize the memory responses induced by this parasite if it is to be used as a vector for other pathogens. Indeed, efforts to engineer safer non-replicating parasites are already underway (Fox and Bzik, 2010), and genetic screens have identified numerous genes associated with virulence in *T. gondii* (Saeij et al., 2006; Taylor et al., 2006). In the following sections, the contributions of two virulence factors, ROP18 and ROP16 are discussed.

ROP 18

One recently identified virulence factor that merits special attention is the secreted kinase ROP18, which provides a major contribution to the virulence of *T. gondii* during murine infection (Saeij et al., 2006; Taylor et al., 2006). As described in the introduction of this thesis, ROP18 directly phosphorylates members of the IRG enzyme family, and thereby prevents the disruption of the parasitophorous vacuole (Fentress et al., 2010; Fentress and Sibley, 2011; Steinfeldt et al., 2010). Although there is evidence that this enzyme is less important for evading immunity when human cells are infected (Niedelman et al., 2012), there may be functional orthologous of ROP18 specialized to phosphorylate immune enzymes in human cells. Deletion of ROP18 or functional orthologes that mediate virulence during human toxoplasmosis may therefore be one strategy to make administering *cpsII* parasites to humans safer. Additionally, ROP18 has been proposed to

inhibit CD8+ T cell responses by binding to the host protein ATF6-β, and deleting ROP18 from *cpsII* parasites may therefore enhance their efficacy (Yamamoto and Takeda, 2012). However, the role of parasite persistence in generating T cell responses and memory following *cpsII* vaccination is not currently known, and deleting ROP18 could therefore have adverse effects on the efficacy of *cpsII* parasites as a vaccine. Interrogating how ROP18 and members of the IRG family affect *cpsII*-induced T cell responses could provide insight into the roles of parasite persistence and vacuolar disruption in generating T cell responses, and therefore facilitate the design of future vaccines.

ROP16

Another virulence factor that may affect the generation of adaptive immune responses is the secreted kinase ROP16, which phosphorylates the signaling molecules STAT3 and STAT6, thus promoting the development of alternatively activated host cells (described earlier in this section) which may inhibit the generation of Th1 immune responses (Butcher et al., 2011; Saeij et al., 2007). While the deletion of this gene could potentially result in greater $CD4^+$ and $CD8^+$ T cell responses, anti-inflammatory cytokines such as IL-10 can also promote memory formation (Cui et al., 2011), and alternatively activated cells can act as a source of this cytokine (Weisser et al., 2013). Further experimentation is required to determine how ROP16 affects both innate cells infected by the parasite, as well as the generation of adaptive immune responses.

Designing attenuated pathogens for vaccination

While *cpsII* parasites may function as a vector for antigen delivery, it may be a more effective strategy to generate other attenuated pathogens for vaccination purposes. While the ability of *cpsII* parasites to induce long-lived protection demonstrates that deleting genes necessary for replication can be an effective strategy for developing other live attenuated vaccines, this strategy may not work for other pathogens. Given the genetic malleability of *T. gondii*, and the ability of the *cpsII* parasites to induce immune responses while controlling for antigenic burden, vaccination with *cpsII* may serve as a convenient system to determine how the deletion of other types of

genes (eg. metabolic genes, structural genes, virulence factors) affects adaptive immune responses. The principles learned from such studies may than be applied to develop live attenuated vaccines for other, less genetically malleable pathogens.

Targeting antigen for T cell responses

The design of effective vaccines that elicit protective $CD8^+$ T cell responses has been a long-standing and elusive goal in the field of vaccinology. If the use of live attenuated *T. gondii* proves to be insufficiently safe for human use, perhaps the properties of *cpsII* parasites that promote the generation of protective T cell responses can be identified and mimicked using other vaccination systems. The finding that phagocytosis of antigen is insufficient to generate T cell responses highlights the need for other mechanisms of antigen delivery. Cytoplasmic delivery of antigen using nanoparticles has been developed and tested in murine models, where it has been an effective strategy for generating T cell responses (Moon et al., 2012). If efferocytosis is found to be the pathway by which antigen is acquired for presentation following *cpsII* vaccination, perhaps targeting antigens or antigen-bearing nanoparticles to the efferocytic pathway would be an effective strategy for generating T cell responses. Identifying the innate sensing mechanisms that may determine whether a cell that efferocytoses foreign antigens induces tolerant or protective T cells may also be useful for vaccine design.

Generating effective T cell memory

Although the focus of this thesis has been on the factors that lead to the generation of $CD4^+$ and $CD8^+$ T cell responses, the generation of protective immunity also requires that long-lived memory cells be maintained following vaccination. Current models suggest memory formation is inhibited by inflammatory signals and antigen persistence (Cui and Kaech, 2010; Kaech and Wherry, 2007) (Joshi et al., 2007; Sarkar et al., 2008), however inflammatory signals are also required to drive T cell responses (Curtsinger and Mescher, 2010). Since the *cpsII* parasites are able to induce sufficient inflammation to generate T cell responses and the formation of memory cells, while avoiding the overt inflammation characteristic of actual infection, they are an ideal

model to study the specific sources of inflammatory stimuli necessary to generate long-lived T cell memory. While IL-12 has been shown to promote the T cell responses induced by *cpsII* vaccination (Wilson et al., 2010; Wilson et al., 2008), the sources of this IL-12 and the inflammatory stimuli that elicit its expression remain unknown. Identifying the cellular sources of IL-12 critical for the induction of T cell responses following *cpsII* vaccination may allow for the design of targeted adjuvants that optimize the development of T cell memory.

Although memory T cells are often understood as T cells that persist in the absence of their cognate antigen (Cui and Kaech, 2010), and persistence of antigen can inhibit the development of memory cells, several studies have demonstrated that persistence of antigen is necessary for optimal protection upon re-encountering a pathogen (Belkaid et al., 2002; Cockburn et al., 2010). Although studies in this thesis and other reports have suggested that viable *cpsII* parasites are cleared following infection (Jordan et al., 2009), it remains to be determined whether antigen depots persist following *cpsII* vaccination. Adoptively transferring CFSE-labeled congenic antigen-specific $CD8^+$ T cells to *cpsII*-vaccinated mice at various times following vaccination, and measuring their proliferation may be useful to determine whether antigen persists following *cpsII* vaccination (Cockburn et al., 2010).

Another interesting characteristic of the *cpsII*-vaccine system is that the $CD8^+$ T cells induced by vaccination retain an effector phenotype (characterized by high expression of KLRG1 and low expression of CD62L and the IL-7R) long after vaccination (Jordan et al., 2009). Current paradigms maintain that during acute infection, CD8+ T cells can be characterized as either short-lived effector cells (which express high levels of KLRG1 and low levels of IL-7R) and memory-precursor effector cells (which express low levels of KLRG1 and high levels of the IL-7R), and memory precursor cells display greater homeostatic turnover, longer half lives, and greater potential to expand upon reinfection (Cui et al., 2011; Joshi et al., 2007; Sarkar et al., 2008). Although memory CD8+ T cells that persist following *cpsII* vaccination gradually shift to a $KLRG1^{LOW}IL\text{-}7R^{HI}$ phenotype, this transition occurs very slowly in contrast to other systems. This is surprising, given that inflammation is thought to promote the development of $KLRG1^{HI}IL\text{-}7R^{LOW}$ cells, and *cpsII* vaccination induces relatively low levels of inflammation. Murine vaccination with

cpsII parasites may therefore be a useful model to identify other factors that regulate the development of short-lived effector cells, and the factors that regulate the transition of the memory pool toward a KLRG1^{LOW}-IL-7R^{HI} phenotype.

It is also worth noting that although central memory T cells (CD62L^{HI}IL-7R^{HI}KLRG1^{LOW}) are thought to confer greater protection than effector cells (CD62L^{LOW}IL-7R^{LOW}KLRG1^{HI}) (Wherry and Ahmed, 2004; Wherry et al., 2003), the protection conferred by *cpsII* vaccination correlates with large pools of effector cell CD8^{+} T cells. Furthermore, although neutralizing IL-12 at the time of vaccination can result in greater expansion of CD8^{+} T cells at the time of challenge (Wilson et al., 2010), the effect that this expansion has on parasite clearance following challenge is unclear, especially given that *cpsII* vaccination of IL-12-deficient mice fails to confer protection (Sukhumavasi et al., 2008). Sorting on KLRG1^{HI} and KLRG1^{LOW} CD8^{+} T cells from vaccinated mice and transferring these cells to recipients that are subsequently challenged with virulent *T. gondii* may be useful to determine which population is responsible for parasite clearance. Additionally, the effects of neutralizing IL-12 during vaccination on the ability of CD8^{+} T cells to clear the parasite at the time of rechallenge should be examined. It may indeed be that during infection with *T. gondii* long-lived effector cells provide greater protection than central memory T cells. Such a finding would have broad impact in the filed of vaccinology, as it would suggest that the ideal phenotype of memory T cells to confer optimal protection may differ among infections with various pathogens.

Conclusions

The lack of effective vaccines that elicit long-lived memory T cells underscores the need to better understand the factors that contribute to the induction of adaptive immune responses. The findings presented in this thesis highlight the prominent role of dendritic cells in the development of protective immunity to *T. gondii* and begin to identify the specific cell populations, and specific interactions between host cells and the parasite, that are required for adaptive immune responses to occur. The studies using mice deficient in the cytokine Flt3L establish Flt3L knockout mice as a useful tool to study the role of dendritic cells during immunity to *T. gondii*, and demonstrate that

while Flt3L is not necessary for the infection-induced expansion of dendritic cells that occurs following infection, or the generation of adaptive immune responses during toxoplasmosis, it is required for sufficient production of IL-12 to protect against this pathogen. The discovery that active invasion is required to generate T cell responses may help to explain why live attenuated vaccines are often an effective strategy, and this finding may facilitate the design of vaccination strategies that mimic live attenuated vaccines but mitigate the potential health hazards associated with them. The use of the dual fluorescent reporter system described herein should also be broadly applicable to tracking the fate of other intracellular pathogens, and may aid in determining how phagocytosed antigens are processed, trafficked and ultimately presented. Future studies should aim to determine whether infected cells can directly prime naïve T cells, what role efferocytosis has in the induction of protective immunity to T. gondii, and further identify the innate sensing mechanisms and inflammatory signals that are ultimately responsible for the development of adaptive immune responses to this pathogen.

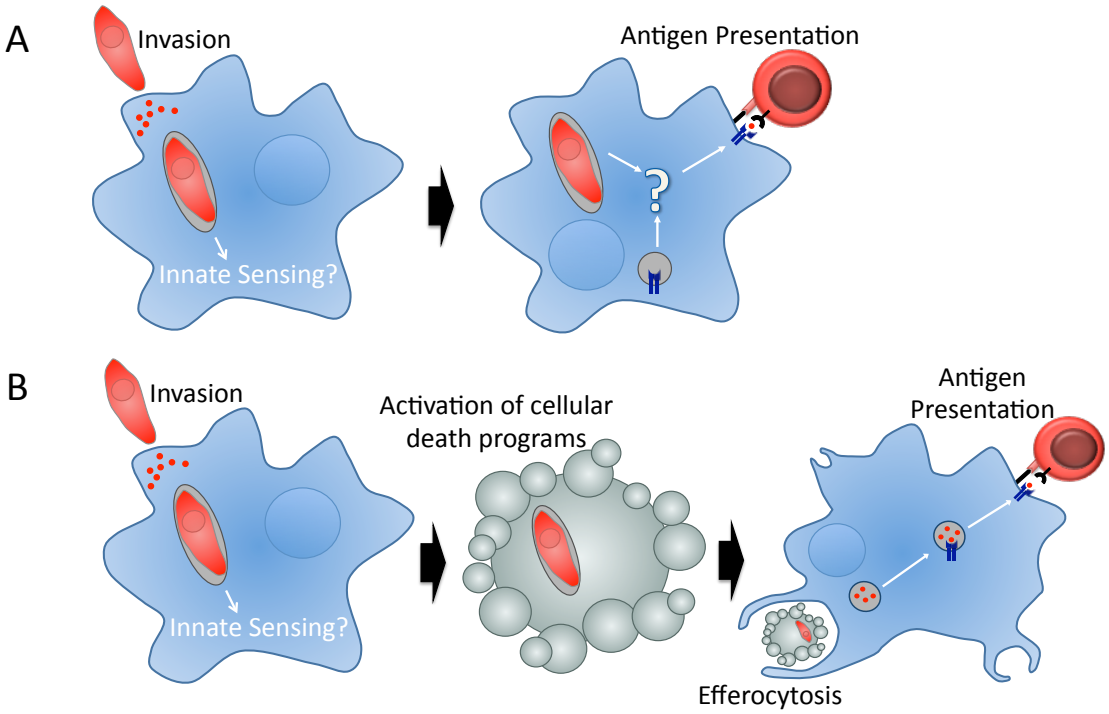

Figure 4.1: Hypothetical pathways of antigen presentation following active invasion. Infected cells may directly present antigen to T cells (A) or may by efferocytosed, leading to antigen presentation by a secondary cell.

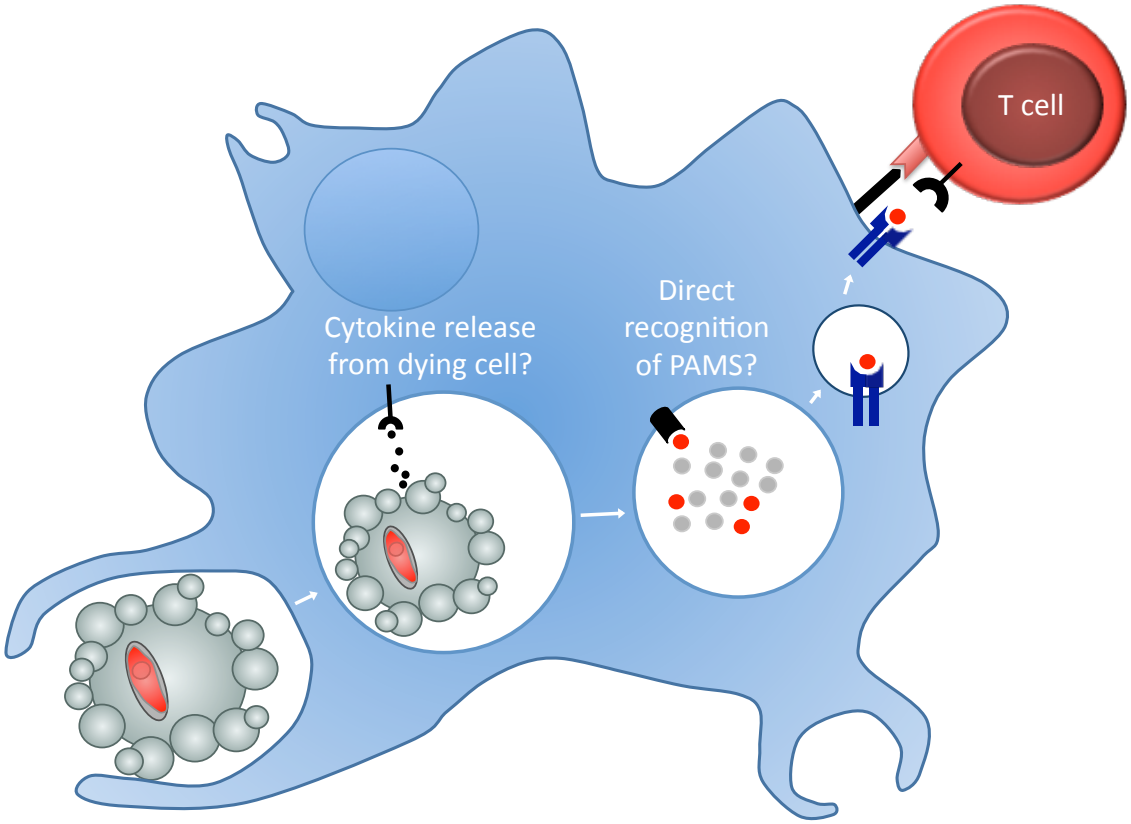

Figure 4.2: Potential mechanisms of sensing foreign antigens in the context of efferocytosis. The infected, apoptotic cell may release inflammatory signals that are sensed by the efferocytosing-cell, or the efferoyctosing-cell may directly recognize the Pathogen-Associated-Molecular-Patterns (PAMS) from *T. gondii* after the breakdown of the cell that is efferocytosed (and the parasite inside of it).

APPENDIX: PUBLICATIONS

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